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Understanding Charged Aerosol Detection With High Performance Liquid Chromatography

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Understanding Charged Aerosol Detection with High Performance Liquid Chromatography

By:

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Dissertation submitted to the Department of Chemistry and Biochemistry of Seton Hall University in partial fulfillment of the requirements for the degree of

DOCTOR of PHILOSOPHY

in

Chemistry

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South Orange, New Jersey

This work is dedicated to my family

My parents:
Mark and Mary-Helen Forsatz

My sister:
Kathryn-Mary Forsatz

We certify that we have read this dissertation and that in our opinion it is sufficient in scientific scope and quality as a dissertation for the degree of Doctor of Philosophy

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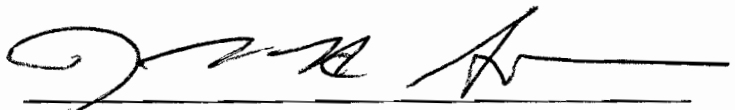

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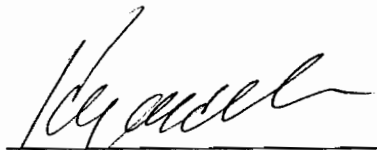

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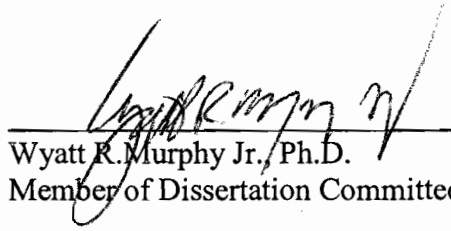

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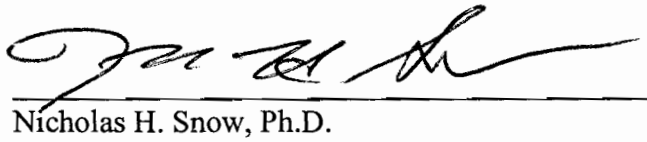

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Abstract

Corona™ charged aerosol detection in high performance liquid chromatography (HPLC) has recently become commercially available, however the detection mechanisms are not well understood. The goal of this research is to better understand this new detection method for HPLC method development by determining the basic chromatographic parameters that have an effect on this detector. These include composition of the mobile phase and its relationship with the baseline signal, the determination of the amount of mobile phase that is associated with detector charging, effects on analyte response and how it they are affected by changes in mobile phase composition, the effects of volatile mobile phase additives, and determination of the actual amount of analyte that is being detected by the electrometer. In addition to developing a better understanding of charged aerosol detection, applications were examined with respect to current detection methods for HPLC, such as ultraviolet (UV) detection. The application that was chosen was Pharmaceutical Cleaning Validation, where HPLC with charged aerosol detection proved to be an acceptable technique for trace level analysis of drug substance on typical pharmaceutical manufacturing equipment surfaces. HPLC-charged aerosol detection was also examined for general pharmaceutical analysis and the ability of this technique to be fully validated to current International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) and United States Pharmacopeia (USP) guidelines. Again HPLC-charged aerosol detection proved itself as an acceptable technique, and

several pharmaceutical assays for in-process control, impurities, and drug substance purity were validated.

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I would like to thank my mentor, Dr. Snow, for taking me on as a graduate student in his research group. His support through my years here at Seton Hall has been the best. I cannot say enough great things about his mentoring. Dr. Snow has not only made me a better scientist, but a better businessman, and better person.

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1. INTRODUCTION

Corona™ charged aerosol detection in high performance liquid chromatography (HPLC) has recently become commercially available, however the detection mechanisms are not well understood. The goal of this research is to better understand this new detection method for HPLC method development by determining the basic chromatographic parameters that have an effect on this detector. In addition to developing a better understanding of charged aerosol detection, applications were examined and compared and contrasted to current detection methods for HPLC, such as ultraviolet (UV) detection.

1.1 Background of Electrical Aerosol Detector

The charged aerosol detector is based on using electrical aerosol analyzer technology **[1-2]**. Electrical aerosol detectors are typically used to assay particulate matter in samples such as dust, diesel exhaust, etc **[3].** It operates by detecting charged particles that have a selected range of mobility rather than by measuring individual gas-phase ions that are differentiated based upon mass to charge ratio as in mass spectrometry **[4].** One of the first direct corona ionizers was made up of a needle electrode upstream of a small sonic orifice which forced almost all of the ions generated by the corona needle through the orifice. This was developed by K. Whitby in 1961 but unlike most particle chargers in aerosol sciences, this device did not directly charge the particles. It used ions as an external source to charge particles in a mixing chamber similar to the charged

aerosol detector **[5].** Over the years this corona-based charger for particles was refined and the mechanism for charging in the charged aerosol detector seen in **Figure 1** was that of the design of TSI Incorporated **[6].** This figure shows the TSI Model 3070A Electrical Aerosol Detector (EAD). This technology is based on diffusion charging of particles which are subsequently detected by a sensitive electrometer.

Examining **Figure 1**, the carrier gas (air) which also contains the particulate matter to be analyzed enters the detector at 2.5 L/minute where it is split such that 40 percent of the flow passes through a carbon filter and then a high efficiency particulate air (HEPA) filter. This air flow is then positively charged using a Corona Needle charging technique which is set at ~2.5kV and forced through an orifice into a mixing chamber. The remaining 60 percent of the flow makes up the aerosol flow. This meets the charged fraction previously separated from the original flow. Through this mixing of the two flows, the aerosol particles are brought into a charged state by diffusion charging. Diffusion charging of air suspended particulates is a technique in aerosol measurements that is well established. The principal mechanism of diffusion charging involves the attachment of charges to the surface of the particles from ions produced by a corona discharge. The sample particles are not directly charged by the high voltage of the corona needle but are charged through a mixing with ions when the two streams of gas flows combine in a mixing chamber **[7-9].** This separation of the corona needle from the aerosol particles minimizes particle losses by

isolating the extreme voltage conditions of ionization from the sample particles. The technique also provides a more efficient, robust, and reproducible process for charging aerosol particles. After the particles are charged they move past the ion trap. A simple low-voltage coaxial precipitator is used to remove excess high-mobility air ions. If this is not done the air ions will contribute to the electrometer measurement. So as to not eliminate actual sample, the electric field and transit time are set so as to not affect particles above 10 nm diameter with a single charge. After passing the ion trap, the particles enter a faraday cage where they are collected on a particle filter. The filter is a conductive metal, and is connected to a sensitive electrometer amplifier. The ions that are attached to the surface of the particles come in contact with the filter and the electric charge they carry is converted to a DC voltage signal in the electrometer amplifier **[10].**

A. Medved found by experimentation that the corona-jet method for charged aerosols resulted in the mean charge per particle being directly proportional to the particle's electrical-mobility diameter, from 10 nm to 1000nm diameter. The net signal seen by the electrometer is proportional to the sum of the diameters of all particles per unit volume. The real performance of this is not ideal, due to having size-dependent losses when the particles move from the charger to the electrometer **[11].**

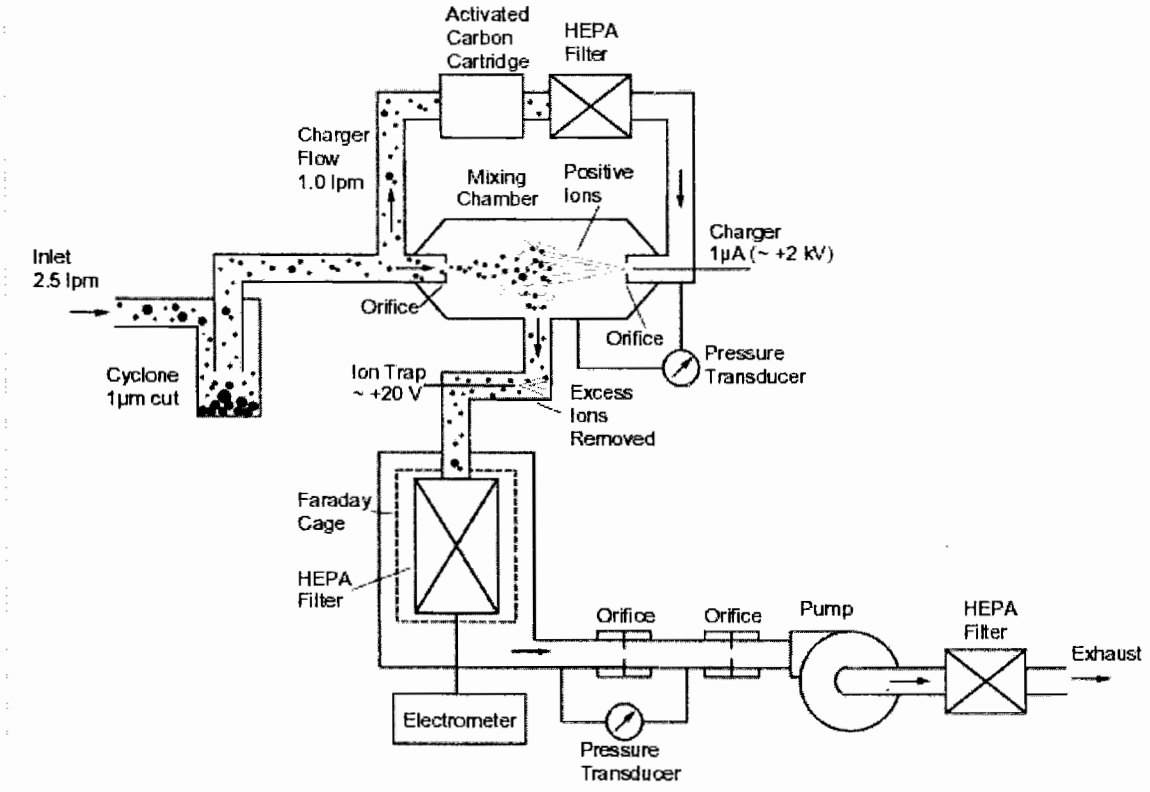


Figure 1: Schematic diagram of electrical aerosol detector, reprinted with permission from TSI Inc.

1.2 Union of Electrical Aerosol Detection with High Performance Liquid Chromatography

The goal of any analytical separation technique is not only to separate all of the compounds of interest, but to be able to detect all of the compounds as well. The choice of the correct detector is dependent upon the properties of the analyte(s) being assayed. Many different detectors are available such as ultraviolet (UV), fluorescence, electrochemical, light scattering, refractive index (RI), flame ionization detection (FID), evaporative light scattering detection (ELSD), mass spectrometric (MS), NMR, and others **[12].** The most common detector that is coupled to high performance liquid chromatography (HPLC) is the ultraviolet-visible spectrophotometer (UV/Vis). These detectors are sensitive, reliable, reproducible, have a wide linear range, and are compatible with mobile phases. However, the UV/Vis detectors do not have a consistency of response for each compound due to the nature of light absorption. Molecules of interest may lack strongly absorbing chromophores with transitions in analytically useful wavelength regions. Specifically not all molecules have chromophores, and molecules that do have chromophores can have varying extinction coefficients at any given wavelength, so the detector response is dependant on the analyte nature. This specificity of UV/Vis detector may present a problem in HPLC method development because the separation may be achievable, but analytes may not be detectable by a UV/Vis. detector.

Certain HPLC methods require the use of a universal detector. A universal detector is one that can see all compounds as they are eluted from the end of the HPLC column with equal sensitivity **[13-14].** Refractive Index detectors are long known universal detectors but have very low sensitivity, and they are not compatible with gradient separations. In the late 1970's and 1980's a detection technique was developed known as evaporative light scattering detection (ELSD) **[15-18].** This was originally hoped to be a universal detector. This detector nebulizes the eluent from the HPLC column, evaporates the droplets, and identifies the aerosoled particles by light scattering. ELSD does not rely on a chromophore as does UV/Vis detection. If the analyte being assayed is not volatile the ELSD is a universal detection method **[19].** Light scattering is a process that is dependent on the size of the particles as shown in **Equation 1**-

$$d_p = d_d(C/\rho_p)^{1/3} \qquad (1)$$

where d_p is the is the droplet diameter, d_d is the particle diameter, C is the concentration, and ρ_p is the density of the particle (given by the density of the analyte) **[20]**. **Figure 2** shows a typical ELSD schematic **[21].** Shown is a simplistic summary of the process: nebulization of the mobile phase, removal of the mobile phase through heating, finally detection. Some models may include a spray chamber to exclude larger aerosol droplets which are problematic due to their relatively slow loss of mobile phase. Unfortunately, the ELSD has several flaws, the most serious of which was that it often gives drastically different responses for compounds that have the same molecular mass. Drastically

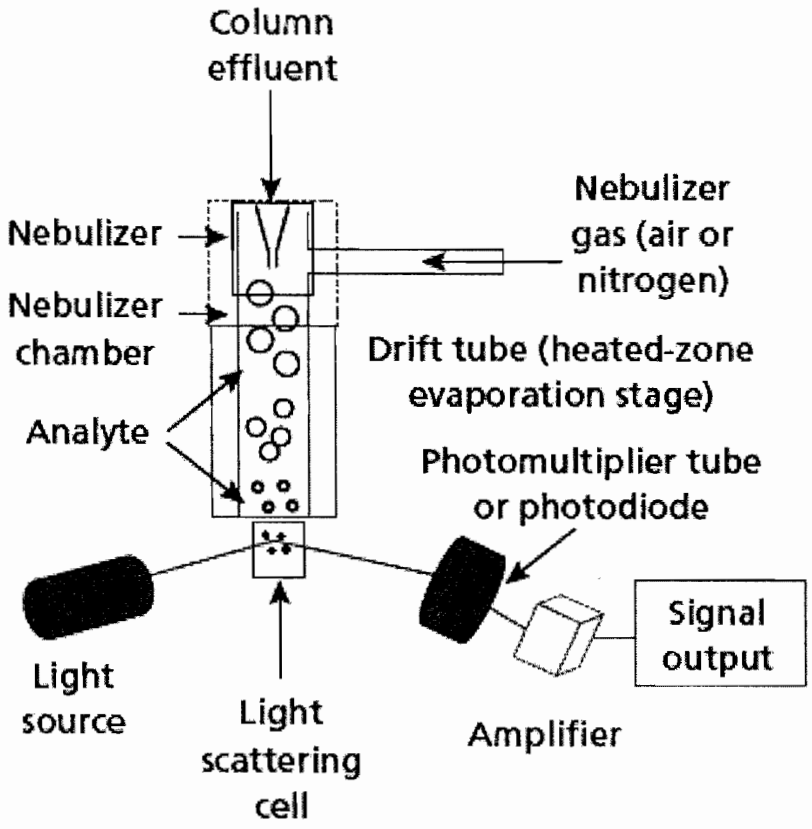


Figure 2: Schematic diagram of evaporative light scattering detector

different responses for compounds with similar molecular masses inhibit the detection of low-level impurities in a compound, and as such the detection technique is generally unreliable.

Dixon and Peterson developed a new more sensitive type of ELSD in 2002 that fixed many of the problems that were present. They coined the term aerosol charge detection for their new version. The particles are not detected by scattered light but by an electrometer which detects an electric charge that has been passed onto the particles by a stream of charged nitrogen gas **[1].**

Figure 3 shows a flow diagram of Dixon and Peterson's aerosol charge detector coupled with an HPLC instrument. After the separation of analytes on the HPLC column the effluent is nebulized using a Meinhard glass nebulizer. A Meinhard nebulizer, as seen in **Figure 4**, has liquid traveling through a capillary which is housed inside the body of the nebulizer **[22-24]**. Surrounding this capillary is the nebulization gas, in this case nitrogen, which travels inside the body of the nebulizer. At the tip of the nebulizer the gas and the liquid meet producing an aerosol. The nitrogen gas used for nebulization was heated in an oven to 50ºC prior to entering the Meinhard glass nebulizer. The aerosol moves into a spray chamber which discriminated some of the larger droplets by spraying them at an impactor, which would subsequently drain into a waste container (**Figure 5**). The remaining aerosol exits the spray chamber and enters once again into the oven at 50ºC before moving into the electrical aerosol detector which Dixon refers to

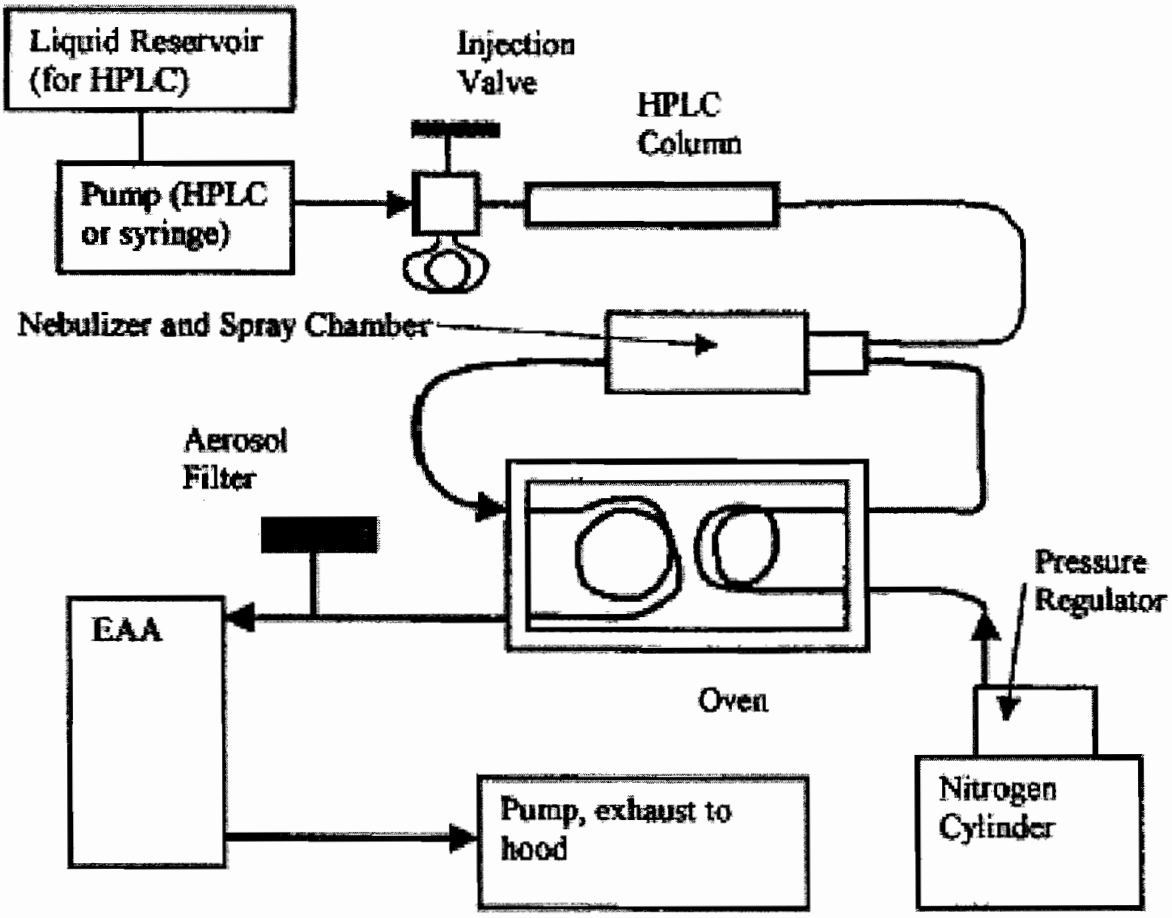


Figure 3: Flow diagram for Dixon and Peterson aerosol charge detection system.

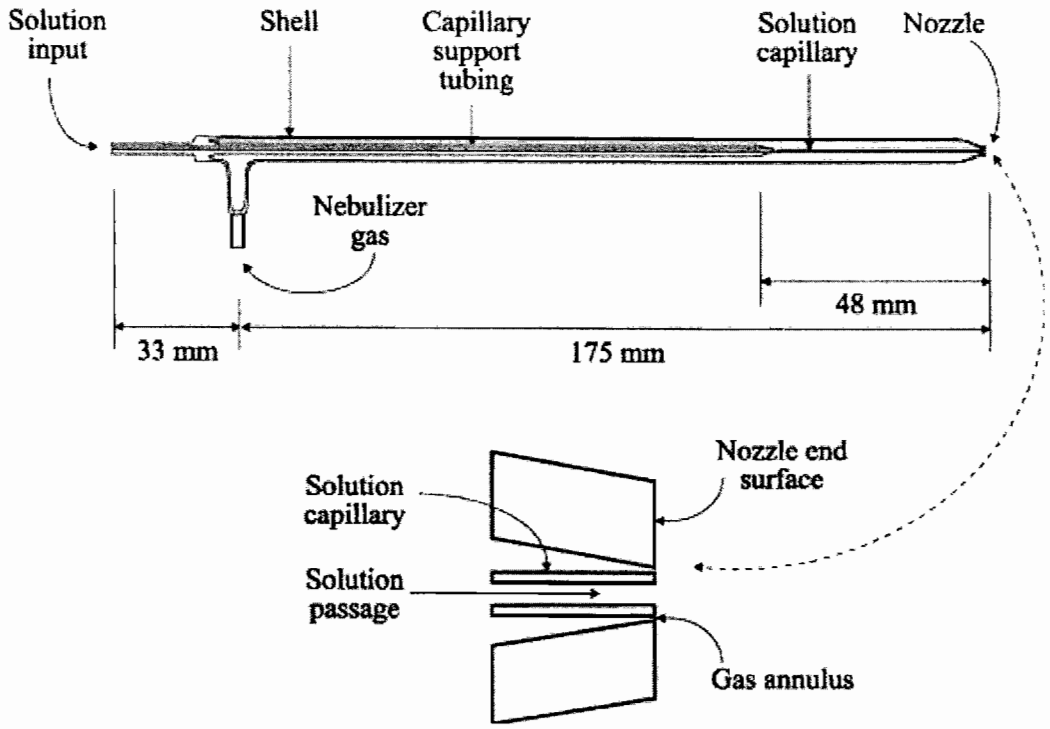


Figure 4: Diagram of a Meinhard nebulizer.

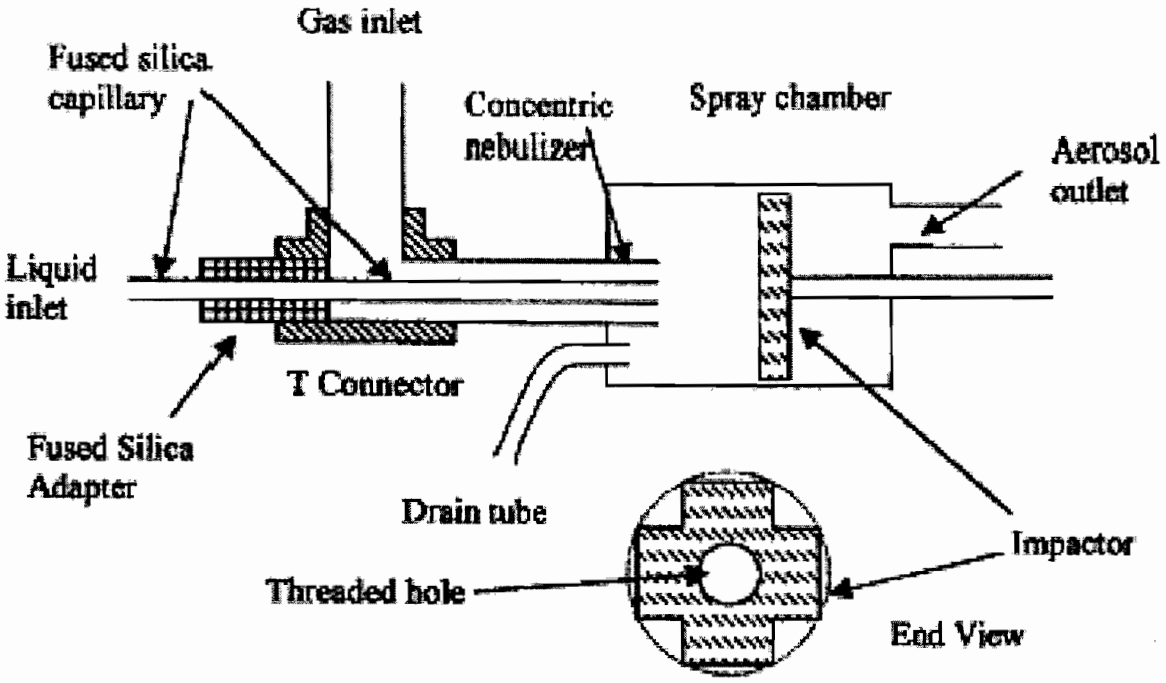


Figure 5: Diagram for Dixon and Peterson capillary nebulizer and spray chamber.

as an electrical aerosol analyzer (EAA). The EAD which Dixon used was the same style from TSI Inc. described in **Figure 1**.

This approach was commercialized by ESA Biosciences in 2004, launched as the Corona™ Charged Aerosol Detector (CAD™). While ESA's charged aerosol detector was similar in principal to Dixon's detector it also differed in certain respects. **Figure 6** shows a flow diagram of ESA Biosciences charged aerosol detector coupled with a HPLC. The carrier gas (nitrogen) enters the detector at 4.0 L/minute where it is split such that 25 percent of the flow passes through a carbon filter and then a HEPA filter.

This gas flow is then positively charged using a Corona Needle charging technique which is set to ~2.5kV and forced through an orifice into a mixing chamber. The remaining 75 percent of the flow containing the aerosol flow which meets the other 25 percent of the original flow, which is now charged. After the separation of analyte on the HPLC column the liquid is nebulized using a cross flow nebulizer.

A cross flow nebulizer, as seen in **Figure 7**, has liquid traveling through a tube which is at a 90º angle to a second tube which contains the nebulization gas, in this case nitrogen. The tips of these two tubes come together in close proximity and the liquid flow is introduced into the flow of the gas to produce the aerosol. Unlike the Dixon design, the charged aerosol detector operates entirely at room temperature (approximately 22ºC) from nebulization to detection. The aerosol moves into a spray chamber which discriminates the larger droplets by spraying

them at an impactor, which would subsequently drain into a waste container, shown in **Figure 8**. This impactor is different in design from that of Dixon. The charged aerosol detector has a hemispherical impactor which is attached to a rod. The aerosol is sprayed directly at the center of the impactor.

Larger droplets that have been produced in the aerosol will hit the impactor and drip to the bottom of the spray chamber where they will travel, by gravity, out of the spray chamber and into a waste bottle. The waste bottle is pressurized with nitrogen by a second tube to allow for smoother drainage of the spray chamber.

Some of the smaller droplets in the aerosol will not collide with the impactor and will travel on to drying tube which is also at room temperature. The length of the drying tube is approximately 10-cm. In the drying tube the droplets, which contain non-volatile analyte, become solid particles through the loss of volatile components to leave a solid particle behind in the drying tube. The droplets, which contain only volatile compounds, will be evaporated in the drying tube. The degree residual volatile mobile phase remains in the particle is debatable. Also it is not known if there is a complete volatilization of the droplets that only contain volatile compounds in the drying tube. Assuming that these droplets of mobile phase are completely volatilized, any such eventually charged volatile compounds of high mobility would be removed by the ion trap and not make it to the detector. After traveling through the drying tube the particles enter the mixing chamber where they are met with the positively charged nitrogen produced by the high voltage corona charger needle. From this point on the charged aerosol

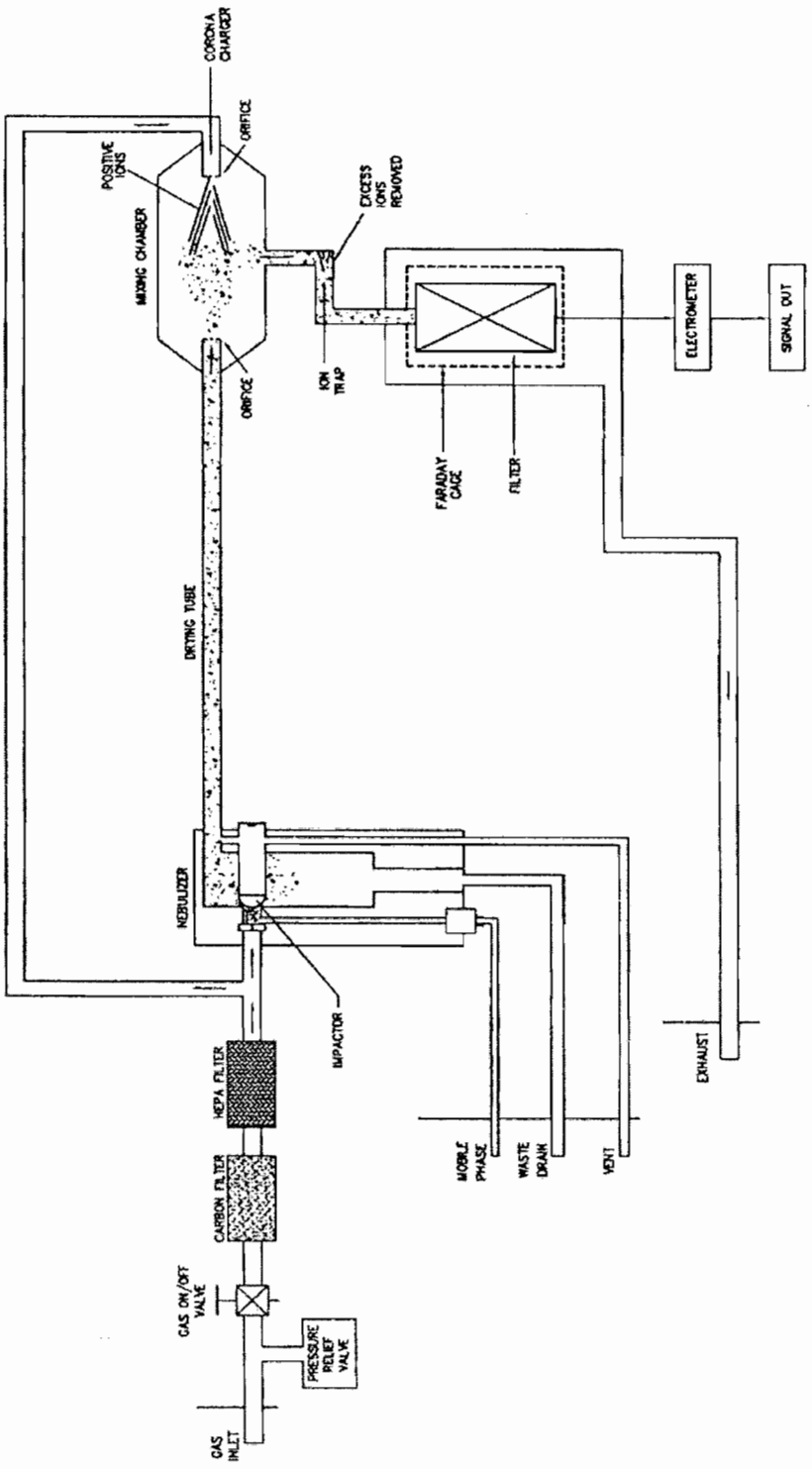


Figure 6: Diagram of ESA Biosciences Corona™ charged aerosol detector, reprinted with permission from ESA Inc.

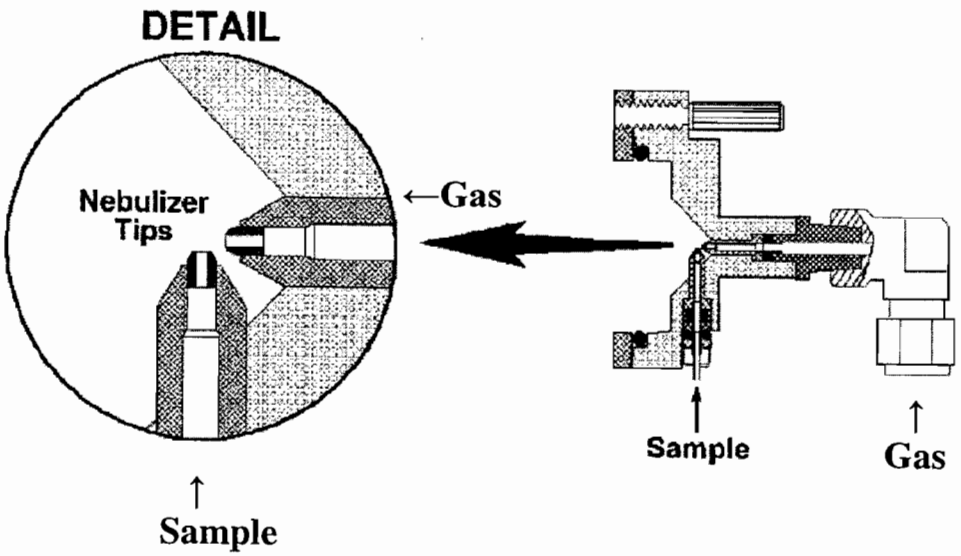


Figure 7: Diagram of a cross flow nebulizer.

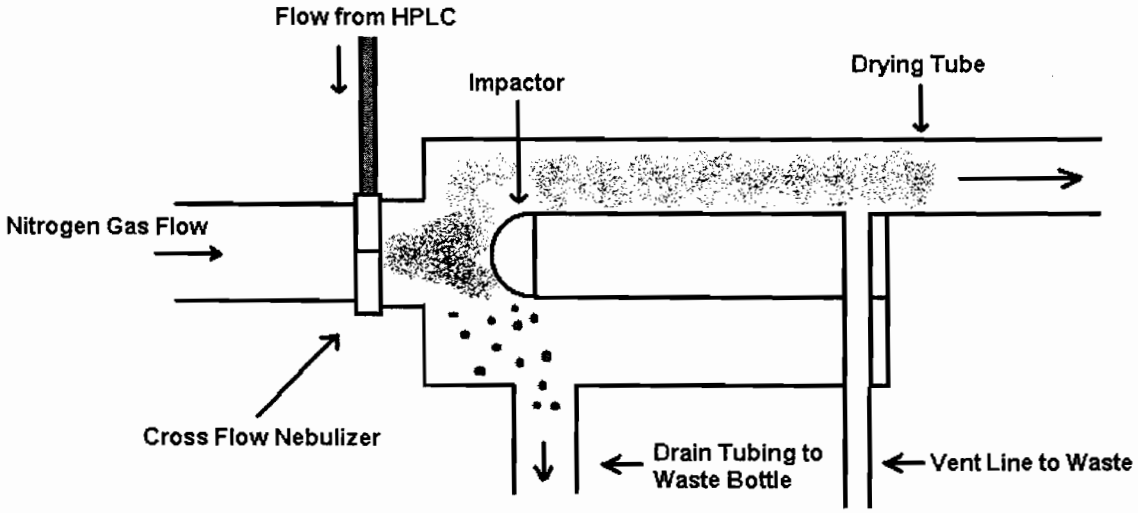


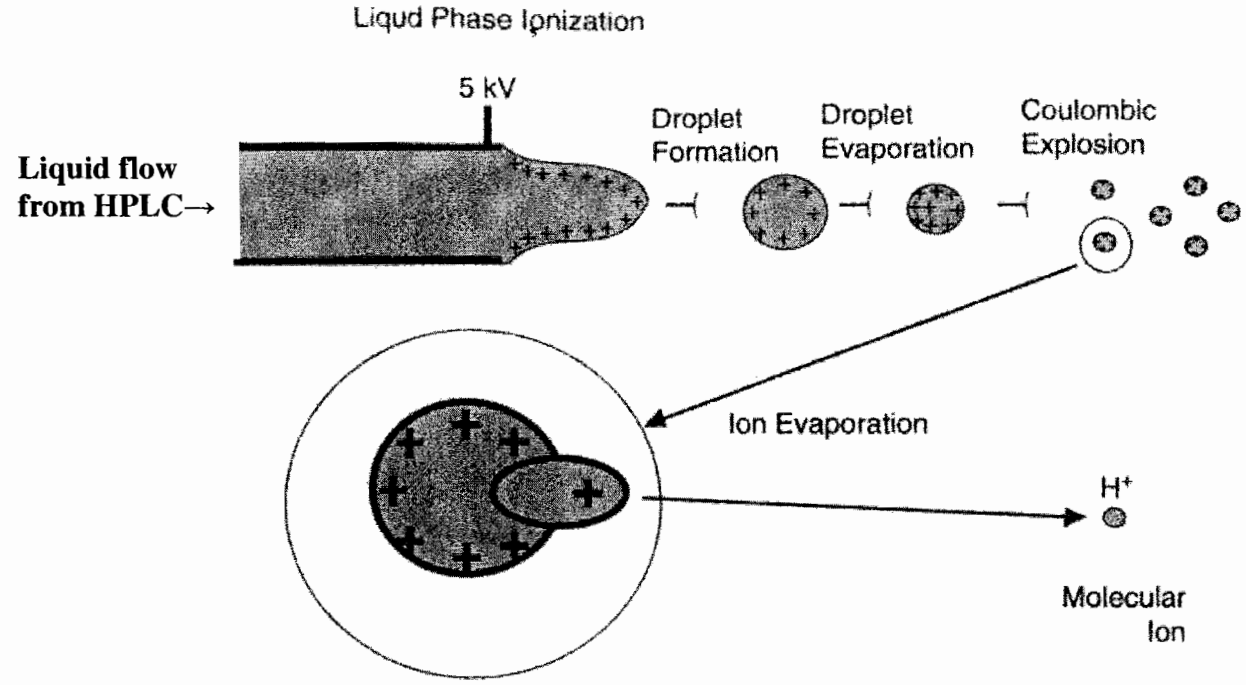
Figure 8: Diagram of impactor in charged aerosol detector.

detector is exactly the same as the EAD from charge diffusion to detection (see Section 1.1 for explanation of charge diffusion to detection).

Since the aerosol droplet and desolvation process for charged aerosol detection is not well known, other types of detection that utilize similar techniques can provide assumptions for what is happening in charged aerosol detection. For example the formation of aerosol droplets, desolvation of those droplets, and the formation of ions in high performance liquid chromatography has been performed for mass spectrometry. This is achieved by the liquid chromatography-mass spectrometry interface, of which many have been developed over the years **[25].** **Figure 9** shows examples of two of these interfaces, electrospray ionization and atmospheric pressure chemical ionization. J. Zeleny made the first description of electrospray ionization in 1917 **[26].** Since then many efforts have been made to further the development of this technique **[27-30].** In the mid-1980's the first use of electrospray ionization was reported independently by M. Yamashita and J. Fenn and M. Aleksandrov **[31-32].**

A basic electrospray interface contains a spray needle at 4-5kV, a thermal/pneumatic desolvation chamber, followed by the vacuum interface. The ionization is of less interest for comparison to charged aerosol detection, however the aerosol droplets that are formed and the removal of solvent is of interest. While electrospray does not have a spray chamber to discriminate larger droplets as in charged aerosol detection, it uses a Meinhard style nebulizer and a lower flow of liquid to generate micro-droplets which can be assumed to be

Electrospray Ionization



Atmospheric Pressure Chemical Ionization

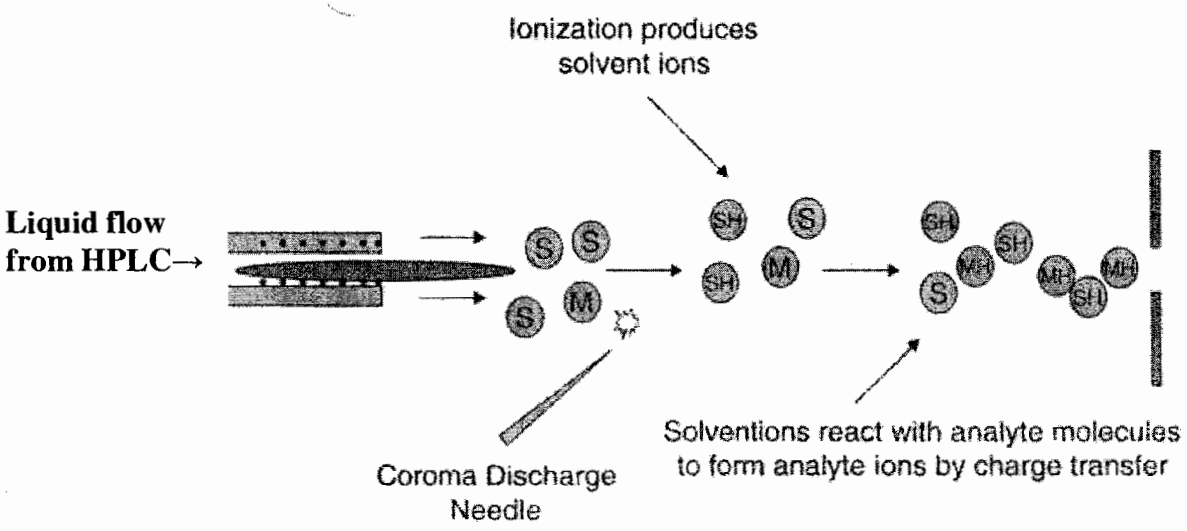


Figure 9: Diagrams of two types of Liquid Chromatography Mass Spectrometry Ionization

similar in size and nature to those produced by charged aerosol detectors post spray-chamber. Electrospray uses either a drying gas (nitrogen) or thermal desolvation to remove the solvent. The drying gas technique is similar to charged aerosol detectors, while the thermal desolvation is not since the charged aerosol detectors drying tubes are at room temperature.

In electrospray the solvent removal is a process that is still debatable. One theory is the Dole/Fenn's coulombic explosion, which is when the surface charge density increases as the droplet becomes desolvated, the coulombic forces exceed the surface tension (Rayleigh stability limit), and the droplets break into smaller droplets. Further evaporation of these smaller droplets with the Rayleigh fragmentation eventually produces the analyte ions **[33].** Dole/Fenn's theory of coulombic explosions is of less interest to charged aerosol detection. However what is of interest is that there is a rather rapid desolvation of the droplets using nitrogen gas which is similar to charged aerosol detection. The desolvation of the droplets in electrospray can be used to assume that there is some desolvation of the droplets in charged aerosol detection moving towards a fully desolvated particle, however the degree of desolvation is debatable.

Atmospheric pressure chemical ionization was developed in the early 1970's by E. Horning **[34],** and is closely related to electrospray. However it is of less interest as a comparison to charged aerosol detection as it uses high temperature (400-500°C) in order to remove the solvent from the droplets. It is notable that the ionization of the particles in atmospheric pressure chemical

ionization is performed through charge transfer similar to charged aerosol detection.

1.3 HPLC Method Validation

In order to determine how useful or applicable the charged aerosol detector could be, many different aspects of that detector needed to be examined. If the detector is to be useful to an analytical chemist it needs to be accurate, precise, robust, sensitive, reproducible, and have a useful range. All of these qualities are covered by one of the most stringent examinations an analytical method can go through, pharmaceutical method validation. Therefore a method was developed for the separation of two pharmaceutical compounds and was subjected to the method validation process. This process should reveal whether this detector is suitable for pharmaceutical use.

In the pharmaceutical industry method validation is required of all analytical methods. These requirements differ depending on which phase of the development process the project is in. A condensed, or simplified, version of method validation is required for the earlier phases up to Phase IIa, after which full method validation is required based on the ICH Guidelines Q2A and Q2B **[35]**. The purpose of the method validation process is demonstration that the method is suitable for its intended purpose. The substance being assayed could be a raw material, drug substance, or a drug product. The assay could range from an identification test, quantitative assay, or a semi-quantitative assay.

Additional requirements for analytical equipment are design qualification (DQ), instillation qualification (IQ), operational qualification (OQ), performance qualification (PQ), and calibration **[36].** Compounds that are being used in the validation, specifically references and impurity markers, should be well characterized and of the highest purity available. Regardless of the purpose of the assay, toxicology, stability, in-process control, clinical release, or finished product release, they all require some form of method validation.

Before executing a full validation of the analytical method, a validation protocol must be written. This method validation protocol may include everything from the exact preparations, and the order in which injections are to be made, or it may be more general. Several points are required in the method validation protocol; the analytical test method for the given drug substance/product, the tests to be validated, the parameters for each test (type and number of solutions, and number of injections), the acceptance criteria, the substances to be used, the reference materials, and the equipment to be used **[37].** The validation protocol needs to be not only fully written, but also fully approved by the appropriate pharmaceutical company's management prior to execution.

Once the protocol is executed, if acceptance criteria are not met for a specific assay a deviation must be added to the validation and a justification will be made. The justification may require a note to the method, re-test of that section of the validation, or a failure of the validation. A failure of the validation would

result in redevelopment of the method. After a method validation is executed a validation report is written which clearly states the validation testing that was performed and the results as compared to the pre-defined acceptance criteria.

The parameters that need to be examined in early stage development can be seen in **Table 1**. The pre-Phase IIa stage parameters are much less involved. A list of full validation parameters are shown in **Table 2**. Both stages of method validation share some of the parameters, with the full validation requiring more parameters depending on the type of assay being validated. For a quantitative HPLC method the parameters that are required are Accuracy, Linearity, Specificity, Precision, Intermediate-Precision, Range, Limit of Detection, Limit of Quantitation, Solution Stability, and Robustness. Accuracy is a test to show agreement between the data point which is reported and the accepted reference value **[38].** This test can be linked with others in order to save time during the validation. Typically the same solutions are used for Accuracy, Precision, Linearity, and Range. Precision, also know as repeatability is performed by one analyst making replicate injections of multiple preparations in order to show that the method can tolerate random errors **[39].** Intermediate-Precision is performed by a second analyst making replicate injections of multiple preparations on a different day and on a different instrument from the first analyst. Intermediate-Precision is performed to account for variability in the analytical assay. This parameter can be determined by the receiving manufacturing site during the validation process in what is called a Co-Validation.

By executing the Intermediate-Precision in a Co-Validation format, this eliminates the need for a formal Method Transfer. A traditional Method Transfer would require that the HPLC method have been fully validated according to the International Council on Harmonisation (ICH), United States Pharmacopeia (USP), and European Pharmacopeia (EP) **[42-45].** Linearity is the ability of the entire system to obtain results that are directly proportional to the concentration of analyte in the sample assayed **[46].** The Range of a method is defines the upper and lower limit of the concentration for an analyte. This Range must also meet the requirements of Precision, Accuracy, and Linearity. Specificity of a given HPLC method requires it to be able to separate the analyte in the presence of impurities, degradants, and/or matrix compounds that may be expected. The requirements of specificity are often determined very early in method development and may be simple or complicated dependent on the number of impurities, degradants, and/or matrix compounds that may be present.

The definition of Limit of Detection (LOD) as per the USP is "a parameter of limit tests. It is the lowest concentration of analyte that can be detected, but necessarily not quantitated, under the stated experimental conditions **[47]**." The chromatographic requirements for the LOD is a signal to noise ratio greater than or equal to 3:1. The definition of Limit of Quantitation (LOQ) as per the USP is "a parameter of quantitative assays for low levels of compounds in sample matrices, such as impurities in bulk drug substances and degradation products in finished pharmaceuticals. The LOQ is the lowest concentration in a sample that may be

Validation Parameters	Type of Tests to Be Validated		
	Identity	Weight Percent/ Assay / Content Uniformity / Dissolution	Impurity Testing: Quantitative Test
Specificity	Yes	Yes	Yes
Linearity	No	Yes	Yes
Accuracy	No	Yes	Yes
Precision (repeatability)	No	Yes	Yes
Limit of detection	No	No	Yes
Limit of quantitation	No	No	Yes
Stability of the solutions	No	Yes	Yes

Table 1: Early stage development validation requirements **[40]**

Validation Parameters	Type of Tests to Be Validated		
	Identity	Weight Percent/ Assay / Content Uniformity / Dissolution	Impurity Testing: Quantitative Test
Specificity	Yes	Yes	Yes
Linearity	No	Yes	Yes
Accuracy	No	Yes	Yes
Precision (repeatability)	No	Yes	Yes
Precision (intermediate)	No	Yes	Yes
Precision (reproducibility)	No	Rarely	Rarely
Range	No	Yes	Yes
Limit of detection	No	No	Yes
Limit of quantitation	No	No	Yes
Stability of the solutions	No	Yes	Yes
Robustness	Maybe	Yes	Yes

Table 2: Full development validation requirements **[41]**

measured with an acceptable level of accuracy and precision, under the stated experimental conditions." The chromatographic requirement for the LOQ is a signal to noise ratio greater than or equal to 10:1 **[47].**

The purpose of Solution Stability is to show that the reference and sample solutions are stable over the course of normal testing conditions **[48].** These time requirements are often less than 24 hours for most HPLC assays, however additional time points are often assayed, some times up to 7 days. Robustness of an analytical method is examined in order to demonstrate that small, but deliberate changes to the methods parameters have a small affect or no affect on the method, and that the method remains reliable over the course of use **[49].**

For any HPLC method all of the aspects for method validation listed in **Table 2** need to be considered during method development in order to ensure that the method is validatable. The validatability of charged aerosol detection has been examined in this work. Aspects of method validation requirements have been tailored towards UV/Vis detection for HPLC methods. The experiments in this body of work have shown where charged aerosol detection meets these requirements, and where an acceptable alternative approach must be taken.

1.4 Pharmaceutical Cleaning Validation

In order to further understand the charged aerosol detector, an industry application was examined. It was decided that the application would be geared

towards the pharmaceutical industry, more specifically its potential use as a tool for cleaning analysis.

Since pharmaceutical cleaning validation typically requires low detection levels, this was a great application to compare and contrast to another standard detection technique to examine the charged aerosol detectors sensitivity.

Good manufacturing practice requires that equipment involved in manufacturing be cleaned to certain low level specifications and that assays and detection limits for confirming equipment cleanliness must be continuously improved. Validation of cleaning processes has long played a critical role in pharmaceutical manufacturing. The United States Food and Drug Administration (FDA) requires that firms have written procedures detailing the cleaning processes used for various pieces of equipment and procedures for validating them **[50].** Typical detection limits described in the pharmaceutical literature are 10 ppm of the analyte or a biological activity level of 1/1000 of the normal therapeutic dose **[51].** Cleaning validation needs a quantitative analytical technique which is generally a separation by HPLC of the drug substance and quantitation of the residual material on the manufacturing device based on a reference standard. S. Harder describes a validated cleaning process as, "A procedure whose effectiveness has been proven by a documented program providing a high degree of assurance that a specific cleaning procedure, when performed appropriately, will consistently clean a particular piece of equipment to a predetermined level of cleanliness." **[52].** Solvents or surfactants (soaps) are the two main types of

cleaning are utilized in the pharmaceutical industry. The assays are developed for isolated intermediate, active pharmaceutical ingredient (API), or the surfactant.

HPLC with UV-Visible detection is commonly used for cleaning validation as well as for traditional assays. HPLC cleaning validation methods are often similar to drug substance assays, and in some cases may be the exact same method. The extent to which the drug substance assay can be used is dependent on the specifications set for the cleaning and the sensitivity of the method. Several techniques can be utilized to increase the sensitivity of the current method such as injecting a higher amount of active pharmaceutical ingredient (API) on the column, choosing a wavelength near 200 nm to increase the signal, using micro-HPLC to obtain a sharper peak (increasing the S/N), or utilizing nonporous silica **[53].** If none of these techniques increase sensitivity, alternate analytical techniques are also used that may offer a higher level of sensitivity. Examples of this are fluorescence detection, ion mobility spectrometry, or charged aerosol detection. Other nonspecific techniques are also utilized in cleaning validation, including gravimetric determination, and total organic carbon assays (TOC).

3. EXPERIMENTAL

3.1 Analytical Method Validation of HPLC-Charged Aerosol Detector

An analytical method was developed for the quantitative assay of a fictional process. The method determines the purity of loratadine (%w/w), and the amount of an impurity mometasone furoate (%w/w). In addition a secondary application was examined by utilizing this method for an in-process control of a fictional reaction completion determination, where the reactant is Mometasone Furoate and the product is Loratadine. Listed below are the method conditions which were chosen on the basis of some method development in order to ensure acceptable separation and retention:

Instrument - Liquid Chromatograph equipped with a Waters 2695 Alliance series solvent delivery system and an ESA Inc. Corona Charged Aerosol Detector.

Detector- 100pA & 35psi Nitrogen

Column- YMC Pack Pro C18, 4.6 x 100mm, 3μm particle size, maintained at a temperature of 40°C

Mobile Phase- A: Water:Acetonitrile (50:50) v/v

B: Water:Acetonitrile (10:90) v/v

Gradient Program-

Time (min.)	Flow (mL/min.)	% Mobile Phase A	% Mobile Phase B	Curve
Initial	1.0	100	0	--
10.0	1.0	0	100	6
10.1	1.0	100	0	11
Note: Equilibrate system back to initial conditions for a minimum of 15 minutes before the next injection				

Injection Volume-	About 5μL
Run Time-	10 minutes
Diluent-	Mobile Phase A
Reference-	Purity: 0.1mg/mL
	Impurity: 0.005mg/mL
Sample-	Purity: 0.1mg/mL
	Impurity: 1.0mg/mL

This method was subsequently subjected to an analytical method validation to the typical criteria used in the pharmaceutical industry. This involved the validation parameters needed for full method development listed previously in **Table 2.**

3.2 Mechanisms of HPLC-Charged Aerosol Detection

The solvents examined were those typical to reverse-phase chromatography-water, methanol, and acetonitrile. The solvents utilized in these experiments were all Fisher Optima Grade. The main focus of this work was to determine how different solvents affect the compounds that are being nebulized, charged, and ultimately detected in the electrometer of the charged aerosol detector.

3.2.1 Charged Aerosol Detection Mobile Phase Background

Before being able to examine how compounds were detected by charged aerosol, a simple and basic understanding of the background response was needed. This was performed by allowing the gradient proportioning valve (GPV)

of the HPLC to mix different %v/v proportions of methanol to water and acetonitrile to water. This was performed by setting the HPLC's gradient program to run in isocratic mode for 15.0 minutes at 100% water then changing instantly at 15.1 minutes to 90:10(v:v) water:acetonitrile which was then run in isocratic mode for 15 minutes. This cycle was repeated until reaching 100% acetonitrile. The type of curve that was run in the program was an instantaneous change (eleven) once the program reached each given time point. **Table 3** illustrates the gradient program ran for both experiments.

3.2.2 Determination of Mobile Phase Associated with Charging

A key issue in how charged aerosol works the rate of the mobile phase. Previously the schematic of the charged aerosol detector have been discussed, and by examining this there are only two places for nebulized mobile phase to end up. The mobile phase is either going to be collected in the waste bottle, or it will continue on to the charging chamber, then to the electrometer, and finally out the exhaust tube. Mobile phases were prepared per **Table 4**. The charged aerosol detector was run under standard conditions- 35 psi of nitrogen, 100 pA range, and ambient temperature. Each mobile phase was primed and allowed to equilibrate on the HPLC and the charged aerosol detector.

After equilibration, the charged aerosol detector was allowed to stay idle for 30 minutes while any residual liquids were allowed to drain or evaporate out of the

Time	Flow (mL/min)	% Water (volume)	% Organic (volume)	Programmed Gradient Curve
0.0	1.0	100	0	11
15.0	1.0	100	0	11
15.1	1.0	90	10	11
30.0	1.0	90	10	11
30.1	1.0	80	20	11
45.0	1.0	80	20	11
45.1	1.0	70	30	11
60.0	1.0	70	30	11
60.1	1.0	60	40	11
75.0	1.0	60	40	11
75.1	1.0	50	50	11
90.0	1.0	50	50	11
90.1	1.0	40	60	11
105.0	1.0	40	60	11
105.1	1.0	30	70	11
120.0	1.0	30	70	11
120.1	1.0	20	80	11
135.0	1.0	20	80	11
135.1	1.0	10	90	11
150.0	1.0	10	90	11
150.1	1.0	0	100	11
165.0	1.0	0	100	11

Table 3: GPV Program for background mobile phase response study

Mobile Phase	Water (mL)	Organic (mL)
1	2000	0
2	1800	200
3	1600	400
4	1400	600
5	1200	800
6	1000	1000
7	800	1200
8	600	1400
9	400	1600
10	200	1800
11	0	2000

Table 4: Preparation of mobile phase for waste bottle analysis

system. The waste bottle was then removed and a new dry waste bottle was placed on the charged aerosol detector. The charged aerosol detector was allowed to equilibrate to the system flow rate and pressure. The HPLC's flow was then set to 1.0 mL/min for 100 minutes as measured by a stopwatch. At 100 minutes the flow was stopped and the drain tube was shaken to drain any liquid that was left in the tube. The waste bottle was then disconnected from the charged aerosol detector and the liquid volume was measured using a 100 mL class B graduated cylinder. This waste liquid was then saved in a sealed bottle for further analysis. The samples collected from the waste bottle experiments were subjected to further analysis. Each liquid sample density was determined by weighing it on a 5 place analytical balance (Mettler Toledo). A 1mL Class A volumetric pipette was used to transfer the sample into a vial that had been previously tared on the balance.

The liquid samples were then assayed for their water content by Karl Fischer Titration using a Mettler DL38 Volumetric KF Titrator. Karl Fischer Titration operates by utilizing a sealed titration vessel. The titrant used was Riedel-de Haen Hydranal®-Comp 5 which consists of an alcohol (ROH), base (B), SO_2 and a known concentration of I_2. Hydranal®-Comp 5 uses methanol as the alcohol, and imidazole as the base. The dilution solvent used in the sealed titration vessel was Fisher Optima Grade methanol. The endpoint for this titration is determined by a potentiometric method. The titrant was standardized using an Aquastar® water reference.

A typical reaction for Karl Fischer titration proceeds as follows:

$$B \cdot I_2 + B \cdot SO_2 + B + H_2O \rightarrow 2BH^+I^- + B^+SO_3^-$$

$$B^+SO_3^- + ROH \rightarrow BH^+ROSO_3^-$$

Prior to a liquid sample being assayed the titration vessel was drained and filled with fresh methanol. The instrument was then allowed to titrate the fresh methanol so that no residual water was present in the vessel. The sample weight was determined by an analytical balance, immediately after which the sample was added to the titration vessel and the volumetric titration began. This process was repeated for each liquid sample collected from the waste bottle experiments.

3.2.3 Response Curve of HPLC-Charged Aerosol Detection

A typical response curve of HPLC-charged aerosol detection was determined by making injections over a wide assay of concentrations. The analyte used for this experiment was loratadine. A YMC Pack Pro C18, 4.6 x 100-mm, 3-μm particle size, maintained at a temperature of 40°C. Mobile Phase A was Water:Acetonitrile (50:50) v/v. Mobile Phase B was Water:Acetonitrile (10:90) v/v. The gradient programs was as follows:

Time (min.)	Flow (mL/min.)	% Mobile Phase A	% Mobile Phase B	Curve
Initial	1.0	100	0	--
10.0	1.0	0	100	6
10.1	1.0	100	0	11

Figure 10 curve 6 shows the linear transition from the initial conditions to the conditions at time 10.0 minutes. Curve 11 is an instantaneous change of conditions at time 10.1 minutes. The injection volume of all samples was 5μL. Run time was 10 minutes with an equilibration time of 15 minutes back to initial conditions before the next injection. The charged aerosol detector was run under standard conditions, 35psi of nitrogen, 100pA range, and ambient temperature. The diluent used for the samples was Mobile Phase A. The solutions were prepared utilizing an analytical balance for weight determination and Class A volumetric pipettes and Class A volumetric flasks for solution dilutions. **Table 5** shows the solutions of loratadine that were prepared.

The effects of mobile phase composition on the response of the HPLC-charged aerosol detection were determined. For this study the only interactions that were observed were those of the sample compounds and the mobile phase. The column was replaced with a length of PEEK tubing for backpressure purposes only. The analytes used for this experiment were loratadine, albuterol, and mometasone furoate. These analytes were each individually prepared in solutions with concentrations at approximately 0.1 mg/mL and 0.05 mg/mL. The mobile phase used was the same as in **Table 4**. The diluent used for each solution varied on the mobile phase specifically, the mobile phase and diluent would always be the same for each condition being assayed.

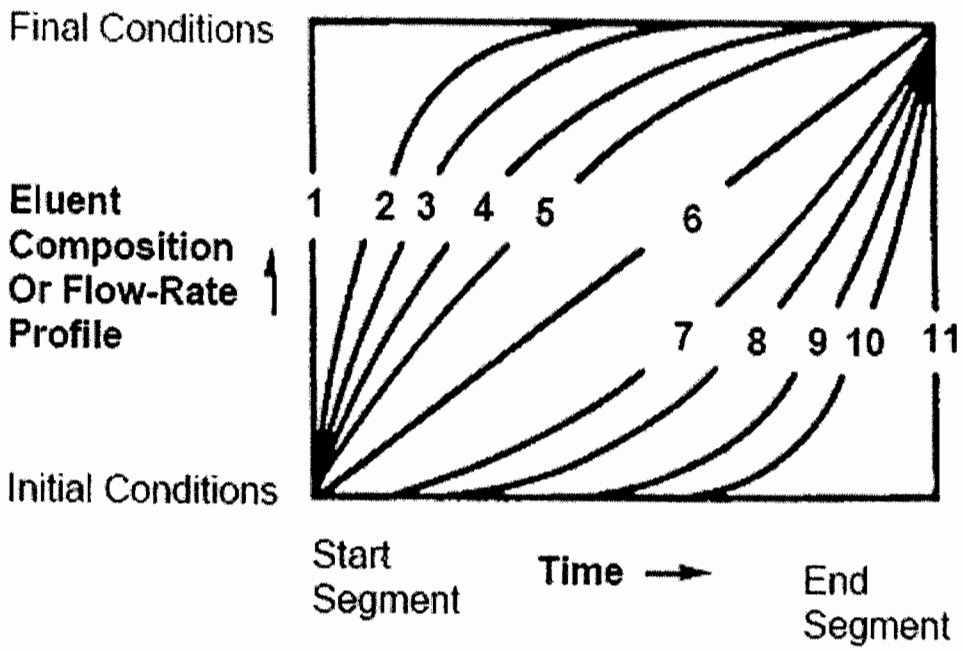


Figure 10: Gradient curve profile for HPLC software **[54].**

Solution Level	Loratadine (ng/mL)
QL	499.6
5%	4996.0
10%	9992.0
25%	24980.0
50%	49960.0
75%	74940.0
100%	99920.0
125%	124900.0
150%	149880.0

Table 5: Prepared loratadine solutions

The injection volume of all samples was 5μL. Run time was 3 minutes with no equilibration time back to initial conditions before the next injection. The solutions were prepared utilizing an analytical balance for weight determination and Class A volumetric pipettes and Class A volumetric flasks for solution dilutions. **Table 6** shows the solutions of loratadine, albuterol, and mometasone furoate that were prepared. The charged aerosol detector was run under standard conditions, 35 psi of nitrogen, 100 pA range, and ambient temperature.

3.2.4 Determination of Analyte Being Detected by Electrometer

The effects of mobile phase composition on HPLC-charged aerosol detection's response were determined by the previous experiment. For this study the amount of analyte that is actually being detected (charged) versus the amount of analyte that is going to waste was examined. The column was replaced with a length of PEEK tubing for backpressure purposes only. No column was used in order to eliminate an additional potential variable, the analyte to stationary phase interactions that might have been taking place. The analytes used for this experiment were loratadine, and mometasone furoate. These analytes were each individually prepared in solutions with concentrations at approximately 0.1 mg/mL. The mobile phase that was use was the same as in **Table 2**. The diluent used for each solution varied on the mobile phase specifically, the mobile phase and diluent would always be the same for each condition being assayed.

The injection volume of all samples was 5 μL. Run time was 3 minutes with no equilibration time back to initial conditions before the next injection. The solutions were prepared utilizing an analytical balance for weight determination and Class A volumetric pipettes and Class A volumetric flasks for solution dilutions. The same solutions from **Table 6** were used. The charged aerosol detector was run under standard conditions, 35 psi of nitrogen, 100 pA range, and ambient temperature.

Each mobile phase was primed and allowed to equilibrate on the HPLC and the charged aerosol detector. The waste bottle was then removed and a new dry waste bottle was placed on the charged aerosol detector. The charged aerosol detector was allowed to equilibrate to the nitrogen flow rate and pressure. The HPLC's flow was set to 1.0mL/min. and allowed to equilibrate for 5 minutes. Ten injections of each solution were made after which the mobile phase was allowed to flow for an additional 5 minutes to flush out any additional analyte that may have been left in the spray chamber or in the drain tubing. The flow was stopped and the drain tube was shaken to drain any liquid that was left in the tube. The waste bottle was then disconnected from the charged aerosol detector and the liquid was poured out into a 100mL graduated cylinder. The waste bottle was rinsed 3 times with small volumes of methanol in order to collect any residual analyte that my have been present. The total volume of the collected waste and rinses were measured using a 100mL graduated cylinder. This waste liquid was then saved in a sealed bottle for further analysis by UV for quantitative

Solution (loratadine, albuterol, mometasone furoate)	Diluent Water:Organic (v/v)	Concentration I (mg/mL)	Concentration II (mg/mL)
1	100:0	0.1	0.05
2	90:10	0.1	0.05
3	80:20	0.1	0.05
4	70:30	0.1	0.05
5	60:40	0.1	0.05
6	50:50	0.1	0.05
7	40:60	0.1	0.05
8	30:70	0.1	0.05
9	20:80	0.1	0.05
10	10:90	0.1	0.05
11	0:100	0.1	0.05

Table 6: Preparation of solutions for mobile phase composition response study

determination. This was performed by taking a fixed volumes of the liquid samples in a vial and evaporating them to dryness under a very light stream of dry nitrogen.

After removing all of the solvents from the sample the residual analyte left in the vial was reconstituted with a small amount of acetonitrile. This, in conjunction with the multiple injections, was done in order to concentrate the low levels of material that was left in the waste bottle. The recovered samples from the waste bottle were assayed by HPLC with UV detection at a wavelength of 250 nm. A YMC Pack Pro C18, 4.6 x 50 mm, 3 μm column was used. The flow rate was 1 mL/min with a 10 μL injection volume and the mobile phase was water:acetonitrile (25:75) v/v. Since the materials that are being used, loratadine and mometasone furoate, are of the highest purity they were used for the quantitative reference as well as for the sample. References were prepared at a concentration of approximately 0.001 mg/mL with acetonitrile as the diluent.

3.3 Properties of Mobile Phase Modifiers with HPLC-Charged Aerosol Detection

A method was developed to examine the possible effect that a mobile phase modifier would have on the charged aerosol detector baseline as well as the analyte signal. The in-process reference solution from the method validation experiments was used in this study. The modifiers that were examined were trifluoroacetic acid, acetic acid, and formic acid at varying concentrations in the mobile phase. Listed below are the method conditions:

Instrument -	Liquid Chromatograph equipped with a Waters 2695 Alliance series solvent delivery system and an ESA Inc. Corona Charged Aerosol Detector.
Detector-	100pA & 35psi Nitrogen
Column-	YMC Pack Pro C18, 4.6 x 50mm, 3μm particle size, maintained at a temperature of 30°C
Mobile Phases-	Water:Acetonitrile (25:75) v/v
	Water:Acetonitrile:Acid (25:75:0.01) v/v/v
	Water:Acetonitrile:Acid (25:75:0.02) v/v/v
	Water:Acetonitrile:Acid (25:75:0.05) v/v/v
	Water:Acetonitrile:Acid (25:75:0.07) v/v/v
	Water:Acetonitrile:Acid (25:75:0.10) v/v/v
Injection Volume-	About 5μL
Run Time-	3 minutes
Diluent-	Mobile Phase A
Reference-	Purity: 0.1mg/mL
	Impurity: 0.005mg/mL

3.4 Using HPLC-Charged Aerosol Detection for Pharmaceutical Cleaning Validation

The HPLC system used was a Waters Alliance 2695 Series HPLC (Waters Corporation, Milford, MA, USA). The system was equipped with a Photodiode Array Detector in line with a Corona Charged Aerosol Detector (ESA Inc.,

Chelmsford, MA, USA). A YMC Pack Pro C18 column, 50 mm X 4.6 mm I.D., 3 μm particle size (YMC Co., Ltd., Kyoto, Japan) was used. Mobile phase was prepared by combining 250 mL of water with 750 mL of methanol. After through mixing the mobile phase was then degassed by vacuum filtration while sonicating.

An orbital shaker (New Brunswick Scientific, Edison, NJ, USA) was used for the agitation of the swab samples. A flow-rate of 1.0 mL/min was used to maintain a reasonable run time. The compounds studied possess different UV absorption profiles therefore different wavelengths were chosen based on the maximum response for detection. Millennium 4.0 (Waters Corporation, Milford, MA, USA) was used to acquire and process the chromatographic data. Sterile cotton balls were prepared for use as swabs by rinsing them with Optima Grade Methanol. A single rinsed cotton ball was then placing into a clean 60 mL bottle. The bottles containing the cotton balls were then placed into a vacuum oven with a slight nitrogen purge to remove the residual methanol from the cotton ball. The bottles were then be removed from the oven and allowed to cool in a desiccator, after cooling they were be capped. Prior to swabbing, 2 mL of acetone were added directly to the cotton ball.

The following were the assay conditions for this study: Mobile Phase- Methanol:Water (75:25); Diluent- Methanol for all three active pharmaceutical ingredients (APIs) and lactose was diluted in Water; Flow- 1 mL/min.; Injection

Volume- 20 μL; Column Temperature- Ambient; Nitrogen Gas – 35 psi; Charged Aerosol Detector Range- 100 pA. The holdup time (t_{o1}) for the HPLC system to the UV detector was determined using the minor disturbance method described by Kazakevich and McNair **[55].** For the charged aerosol detector the holdup time (t_{o2}) was determined by the minor disturbance method plus the difference in retention from the UV to charged aerosol detector of a low level injection of mometasone furoate, (see **Figure 11).**

Minor Disturbance Method for System to UV Detector (t_{o1}) = 0.620min.

Charged Aerosol Detector Retention = 2.190min

UV Retention = 2.167min

Charged Aerosol Detector Holdup Time (t_{o2}) =

(2.190-2.167) + 0.620 = 0.643 min

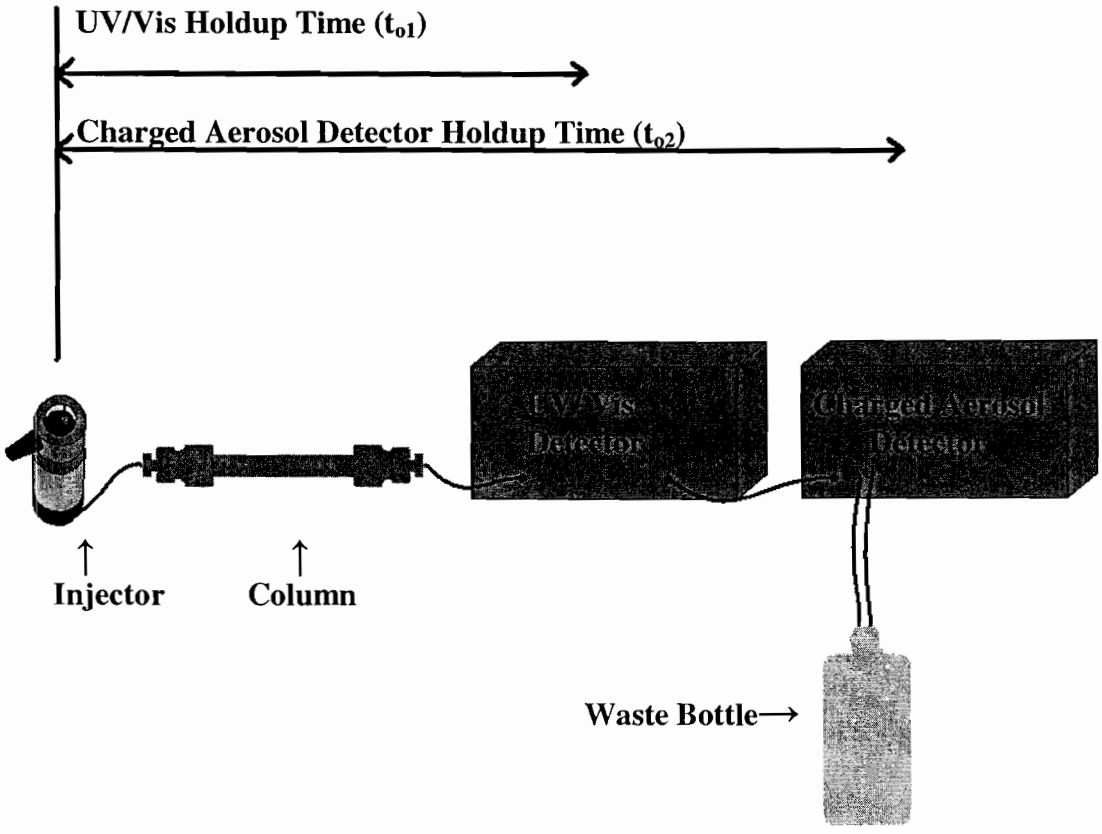


Figure 11: Diagram of the HPLC set up with UV/Vis detector in series with the charged aerosol detector

4. RESULTS AND DISCUSSION

The goal of this research is to better understand this new detection method for HPLC by determining the basic chromatographic parameters that affect this detector. These include composition of the mobile phase and its relationship with the baseline signal, the determination of the amount of mobile phase that is associated with detector charging, effects on analyte response and how they are affected by changes in mobile phase composition, the effects of volatile mobile phase additives, and determination of the actual amount of analyte that is being detected by the electrometer. In addition to developing a better understanding of charged aerosol detection, applications were examined with respect to current detection methods for HPLC, such as ultraviolet (UV) detection. The application chosen was Pharmaceutical Cleaning Validation, of which HPLC with charged aerosol detection proved to be an acceptable technique for trace level analysis of drug substance on typical pharmaceutical manufacturing equipment surfaces. The cleaning validation study was actually the first set of experiments done with the charged aerosol detector in order to gain a practical understanding of the technique while gaining valuable insight into sensitivity. Sensitivity of the charged aerosol detector is of importance since the primary focus on cleaning validation is low-level detection and quantitation. In order to test the characteristics that make up a good detector (accuracy, precision, robustness, sensitivity, etc.) the charged aerosol detector was tested by subjection to one of the most stringent examinations an analytical technique can go through, pharmaceutical method validation. HPLC-charged aerosol detection was fully

validated to current International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) and United States Pharmacopeia (USP) guidelines showing that it is a useful and practical form of detection for high performance liquid chromatography.

4.1 Analytical Method Validation of HPLC-Charged Aerosol Detection

With any new technology there is a period of evaluation which trends towards practical applications and ultimately to a state of acceptance as a valid and robust analytical technique. The charged aerosol detector is no different. Since its commercialization in the fourth quarter of 2004 industry has been developing many practical applications and is in the process of trending towards a state of acceptance as a valid analytical detector for HPLC **[56].** One of the major industries that this new technology has been utilized in is the pharmaceutical industry. This industry is a great test for this new form of detection. Since the pharmaceutical industry is highly regulated, it has strict rules and requirements for any analytical technique/method that is used on any of the products. These requirements for pharmaceutical method validation were applied to a HPLC-charged aerosol detection method to determine if this form of detection was a truly valid and robust analytical technique.

4.1.1 Specificity

For the method that was developed there were only two compounds present, loratadine and mometasone furoate. Loratadine was chosen as the main (API)

compound and mometasone furoate was selected as a hypothetical impurity present from one of the in process reactions. **Figure 12** depicts the typical chromatographic profile that can be expected for this separation. Notice that the impurity peak is well separated from the main peak, and since control of this mock process was in the hands of the designer, there can be confidence that there is not another peak under the main API peak.

4.1.2 Limit of Detection / Limit of Quantitation

Before starting the other sections of a method validation such as Accuracy or Linearity, it is necessary to know what the Limit of Detection (LOD) and Limit of Quantitation (LOQ) are. The LOQ is actually more important to know than the LOD, as the LOQ will be assessed in the other sections of the validation while the LOD will not be assessed. The criteria that needs to be achieved for LOD is a signal to noise of 3 to 1. A signal to noise of 10 to 1 must be achieved for the LOQ. Each company may have different policies governing how limits are determined with regards to chromatography. Some companies may do a manual determination of this by zooming in on the low level peak and measuring the peaks height compared to the baseline noise with a ruler. Other companies may utilize the chromatographic software's signal to noise function to calculate the values. For the purpose of this work the software was used to calculate the signal to noise for this charged aerosol detection method validation. **Figure 13** shows the limit of detection for loratadine. The software calculated the signal to noise to be 3.388 which rounds to 3, and has a solution concentration of

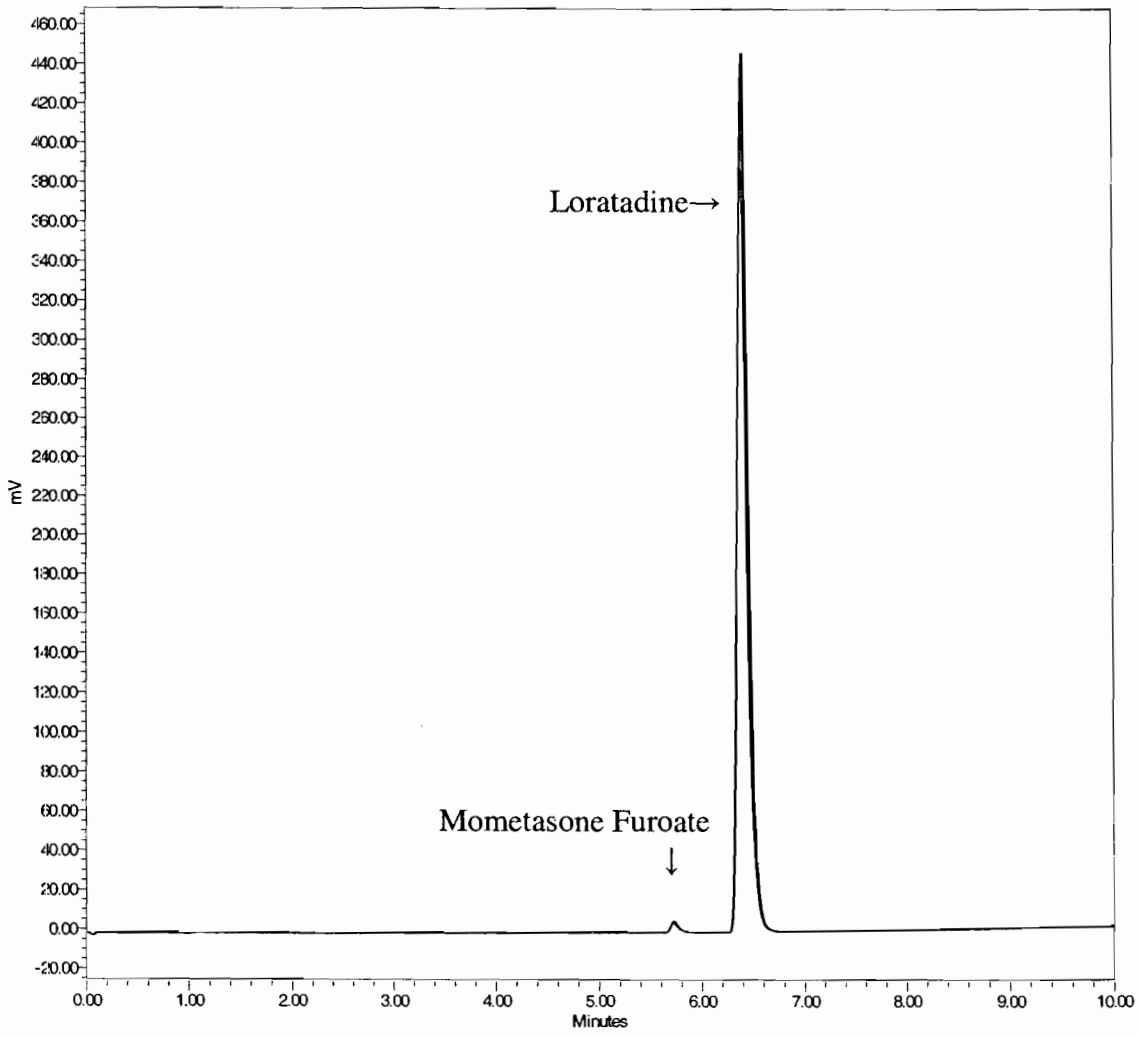


Figure 12 : Chromatography from charged aerosol detector showing specificity for the two compounds that were separated

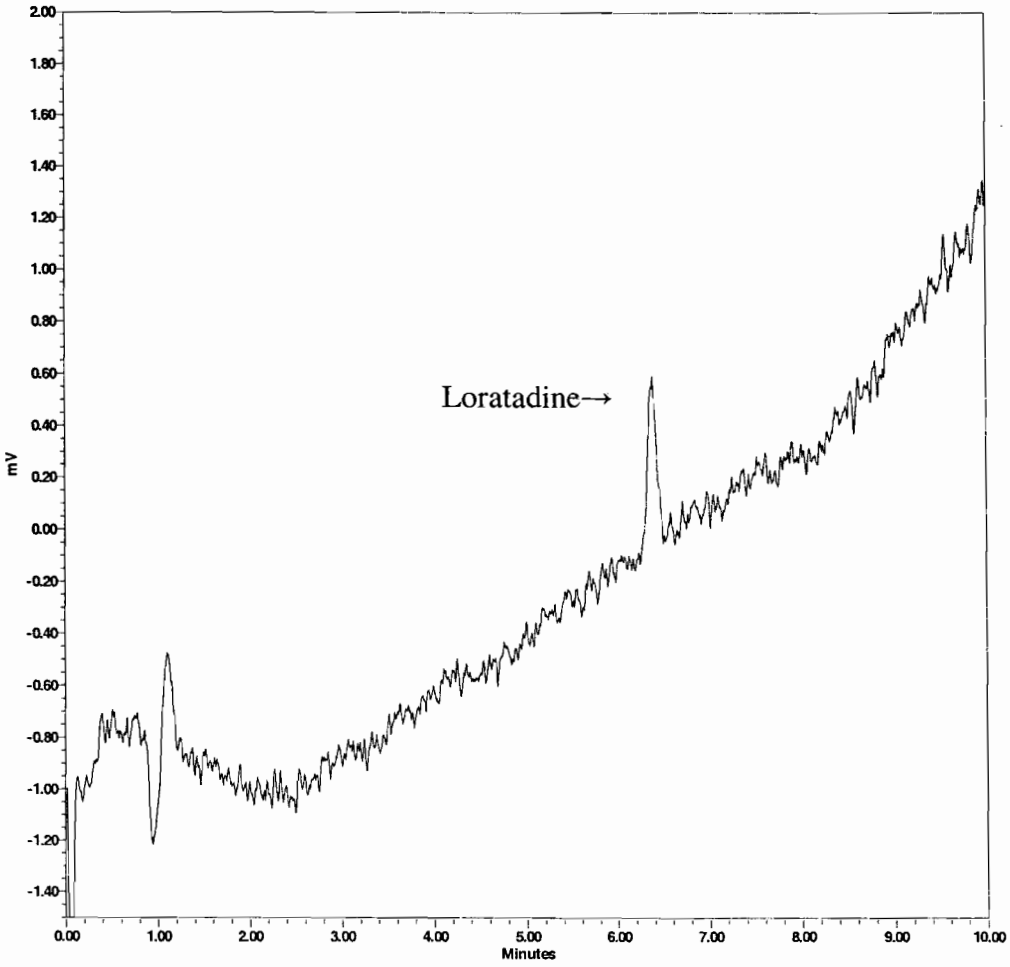


Figure 13 : Chromatography of the limit of detection

0.1499 μg/mL. **Figure 14** shows the limit of quantitation for loratadine. The software calculated the signal to noise to be 10.290 which rounds to 10, and has a solution concentration of 0.4996 μg/mL.

4.1.3 Accuracy

For the purpose of accuracy several different quantitation models were examined. Since the charged aerosol detector is a non-linear detector some compromises must be made to the analytical method. The impurity peak, mometasone furoate, cannot be quantitated against the same reference solution as the main API peak, loratadine. The reason for this is that the concentration of the reference is going to be around the same concentration as the sample, this means that with a non-linear curve the lower concentration impurities will have in accurate results when they are quantitated versus this reference. A simple solution to this is to have a second reference that is at a lower concentration more similar to that of the impurities that are present in the assay. This method was designed in just such a way for the charged aerosol detector, and has been taken into account for the method validation. There is a higher concentration level that will be validated for the main API peak, and a lower concentration level that will be validated for the impurity peak. As previously discussed a linear distribution can be forced over any curve with acceptable results as long as the range is narrow enough. For the high concentration level of this validation this will be done. A narrow range 100 ± 10% of the target concentration was used for the forced linear distribution. A second way was used as a comparison, this was

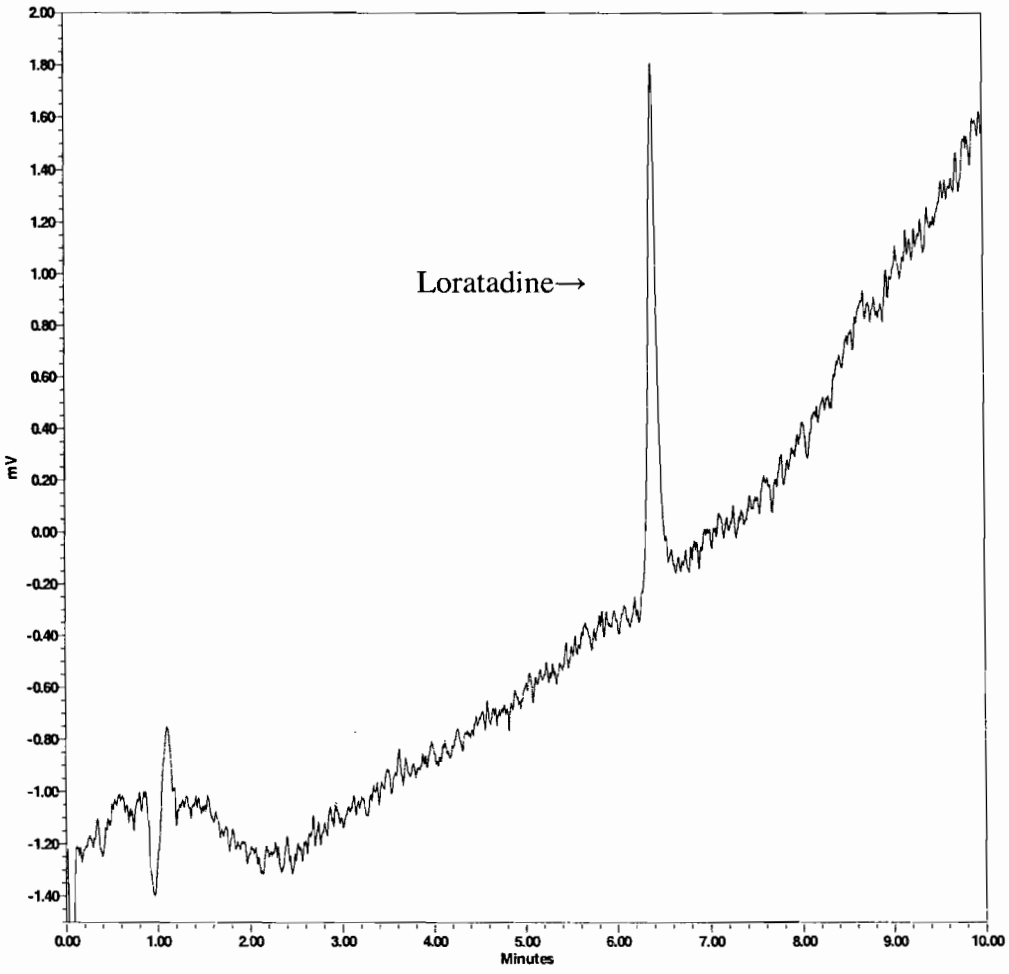


Figure 14 : Chromatography of the limit of quantitation

a wider range 100 ± 50% and a quadratic function was used rather than the linear. For Accuracy of the impurity assay a range from QL to 5.0% of the sample concentration with a linear curve was used. The results from the low level accuracy can be seen in **Table 7.** In the method that is being validated the impurity sample concentration is 1.0 mg/mL. Loratadine has a higher solubility than this and if needed the method could handle a higher concentration injection in order to increase the LOQ; however a LOQ of 0.05 %w/w was achieved and was deemed an adequate LOQ for this method. An acceptance criteria for recovery was set at 100.0 ± 30.0%, which was easily met at each level for each of the three replicate solutions.

For the higher level Accuracy with the narrow range 100 ± 10% of the target concentration and a forced linear distribution the results can be seen in **Table 8**. The calibration used a single point calibration at the 100% concentration to quantitate all of the other solutions. With the narrower range the linear curve yields acceptable results for the high level Accuracy section of the validation. The acceptance criteria was set at 100.0 ± 5.0%, and was met, however it did come close to failing at the extreme ends of the range. This is something that will happen time and time again when using a linear curve for calibration of a charged aerosol detection method when the actual response is not linear.

Using a quadratic curve for calibration was also used for the Accuracy of the high level, the results of which can be seen in **Table 9**. The formula of the

Solution Level	LOR (mg/mL)	Solution #1(Area)	Solution #2(Area)	Solution #3(Area)	Mean (Area)	Solution #1 (%Recovered)	Solution #2 (%Recovered)	Solution #3 (%Recovered)	Mean	Accuracy Acceptance Criteria (%)
QL	0.0004996	11723	12508	11984	12072	80.60	86.00	82.39	83.0	100.0 ± 30.0
0.5%	0.0049960	142813	145532	144358	144235	98.19	100.06	99.25	99.2	100.0 ± 30.0
1.0%	0.0099920	306778	307814	307852	307481	105.46	105.82	105.83	105.7	100.0 ± 30.0
2.5%	0.0249800	806837	805641	795533	802671	110.95	110.78	109.39	110.4	100.0 ± 30.0
5.0%	0.0499600	1611642	1587462	1607904	1602336	110.81	109.14	110.55	110.2	100.0 ± 30.0

Table 7: Accuracy results for low level

Solution Level	LOR (mg/mL)	Solution #1 (Area)	Solution #2 (Area)	Solution #3 (Area)	Mean (Area)	Solution #1 % Recovered	Solution #2 % Recovered	Solution #3 % Recovered	Mean	Accuracy Acceptance Criteria (%)
90%	0.0899280	2600928	2540497	2620476	2587301	99.35	97.04	100.09	98.8	100.0 ± 5.0
95%	0.0949240	2723768	2726871	2695458	2715366	98.56	98.68	97.54	98.3	100.0 ± 5.0
100%	0.0999200	2849640	2840564	2792327	2827510	97.96	97.65	95.99	97.2	100.0 ± 5.0
105%	0.1049160	2915030	2946206	2938180	2933139	95.44	96.46	96.20	96.0	100.0 ± 5.0
110%	0.1099120	3052996	3053231	3050540	3052255	95.41	95.42	95.33	95.4	100.0 ± 5.0

Table 8: Accuracy results for high level using a linear representation

Solution Level	LOR (mg/mL)	Solution #1(Area)	Solution #2(Area)	Solution #3(Area)	Mean (Area)	Solution #1 % Recovered	Solution #2 % Recovered	Solution #3 % Recovered	Mean	Accuracy Acceptance Criteria (%)
50%	0.0499600	1611642	1587462	1607904	1602336	100.56	98.76	100.28	99.9	100.0 ± 5.0
75%	0.0749400	2231480	2246255	2263587	2247107	99.35	100.15	101.10	100.2	100.0 ± 5.0
100%	0.0999200	2849640	2840564	2792327	2827510	100.98	100.57	98.43	100.0	100.0 ± 5.0
125%	0.1249000	3357870	3350030	3389364	3365754	99.57	99.27	100.77	99.9	100.0 ± 5.0
150%	0.1498800	3867718	3867643	3897679	3877680	99.72	99.72	100.74	100.1	100.0 ± 5.0

Table 9: Accuracy results for high level using a quadratic representation

calibration curve was $y = -35239295.4357x^2 + 29737715.0099x + 208493.0037$ and gave an r^2 of 1.000.

With the quadratic curve, acceptable results for the high level Accuracy section of the validation are achieved. The acceptance criteria was set at 100.0 ± 5.0%, and was met, and did not come close to failing at the extreme ends of the range. In addition the range of the quadratic curve was extended well beyond the range of the linear calibration, and gave more accurate recovery results. When using quadratic calibration with the charged aerosol detector a more accurate method calibration with a larger range to work in is obtained. In some industries, such as the pharmaceutical industry, there might be restrictions on what functionality of the software package can be used and the narrower range with a linear model might be the better choice for the circumstances.

For in-process controls that are monitored by HPLC, such as a reaction completion, typically use % normalized area as the reported value.

% normalized area =

$$\frac{\text{Area of Impurity}}{\text{Area of Impurity + Area of Main Peak}} \times 100\% \qquad (2)$$

In order to validate such a method, the work done above is an acceptable way of showing accuracy. However another method to show accuracy is to examine the % normalized area values and to establish criteria. Since a method to monitor a in-process reaction completion is similar to an impurity assay, the same criteria

for Accuracy will be used. In **Table 10** the results for Accuracy in terms of % normalized area are shown. The % normalized areas were compared to the true %w/w values at each level and the % Recovered was determined.

Despite passing the acceptance criteria, this range is not the most acceptable for this specific assay. The deviation from the true values can be seen easily at the 50% level where the true %w/w is 50.00 % and the average % normalized area was 35.99%. For an in-process control, typically the acceptance criteria would not need to be up to the level that was validated in this example. A more realistic range for an in-process control to be validated would be QL to 5%.

4.1.4 Linearity & Range

The linearity data was generated from the multiple solutions assayed from the accuracy section. This is a common practice and saves time and resources. For the determination of linearity for the charged aerosol detector validation, most of the assays will not have any issues and can be treated normally. This includes the impurity assay, the narrow range linear purity assay, and the in-process control assay. However, one of the examples from the accuracy section will not fit into traditional validation criteria, and that would be the quadratic curve for the purity assay. Simply by the type of fit the assay being quadratic does not lend itself to any linearity acceptance criteria. The answer to what needs to be examined is simple - it is the same criteria as with a traditional validation for linearity, r^2. All that is being validated in the linearity section is how well the data

Solution Level	Impurity (%w/w)	Solution #1 (%norm. area)	Solution #2 (%norm. area)	Solution #3 (%norm. area)	Mean (%norm. area)	Solution #1 (%Recovered)	Solution #2 (%Recovered)	Solution #3 (%Recovered)	Mean	Accuracy Acceptance Criteria (%)
QL	0.50	0.410	0.437	0.419	0.42	81.94	87.40	83.75	84.4	100.0 ± 30.0
5%	5.00	4.772	4.859	4.822	4.82	95.45	97.18	96.43	96.4	100.0 ± 30.0
10%	10.00	9.719	9.749	9.750	9.74	97.19	97.49	97.50	97.4	100.0 ± 30.0
25%	25.00	22.066	22.040	21.824	21.98	88.26	88.16	87.30	87.9	100.0 ± 30.0
50%	50.00	36.125	35.777	36.072	35.99	72.25	71.55	72.14	72.0	100.0 ± 30.0

Table 10: Accuracy results for % normalized area

points fit to the curve that is generated by the software. That is exactly what was done for the quadratic curve in this validation - how well the data points fit to the curve that the software generated. This is rather simple for a validation, but there are some differences to the actual test method for a routine assay. Multiple references at different levels would need to be run to generate the calibration curve before assaying the sample. After the sample is assayed check reference solutions would need to be assayed to ensure the system stability during the assay of the samples. This is different than the traditional bracketing with a single reference standard and meeting % RSD criteria for all of the bracketed injections around the sample. Despite being different, the check reference solutions do show system suitability and over all control of the assay.

For the impurity assay, the linearity results can be seen in **Figure 15**. The r^2 acceptance criteria was ≥ 0.9500, and the result obtained was 0.9999. At this low level with the range being small the linearity acceptance criteria were easily met. For the purity assay, the linearity results can be seen in **Figures 16 & 17**. The r^2 acceptance criteria was ≥ 0.9900, and the result obtained was 0.9960 for the wider range (50 to 150%). Despite the linearity passing the criteria, this is not a range that could be validated due to the failure of the Accuracy results over this range. This data was included to illustrate that even if the curve is linear, that the data over that range can be inaccurate, and therefore fail that portion of a validation.

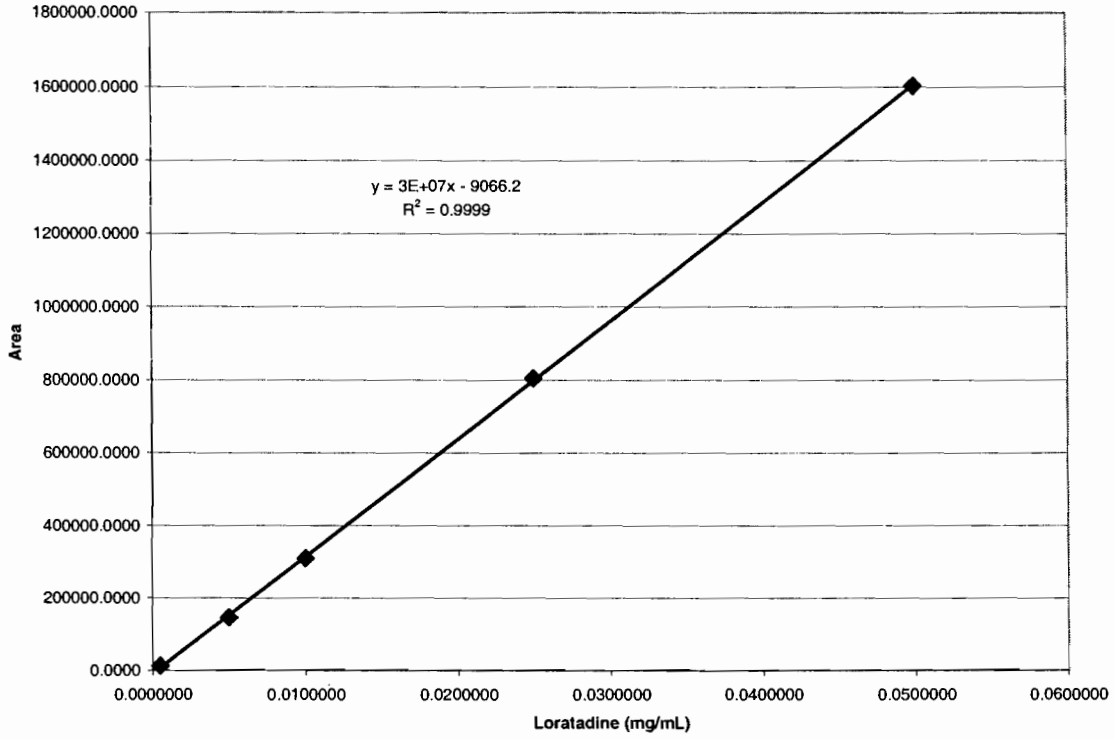


Figure 15: Impurity linearity for charged aerosol detection validation

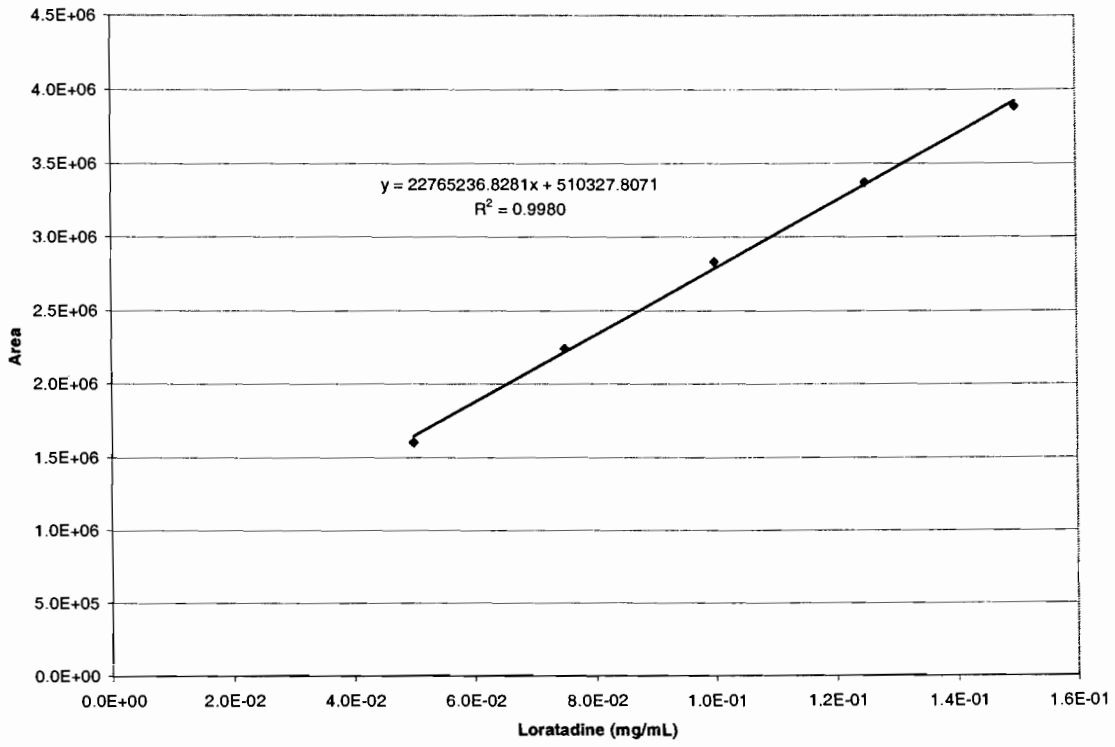


Figure 16: Purity linearity for charged aerosol detection validation from 50 to 150% range

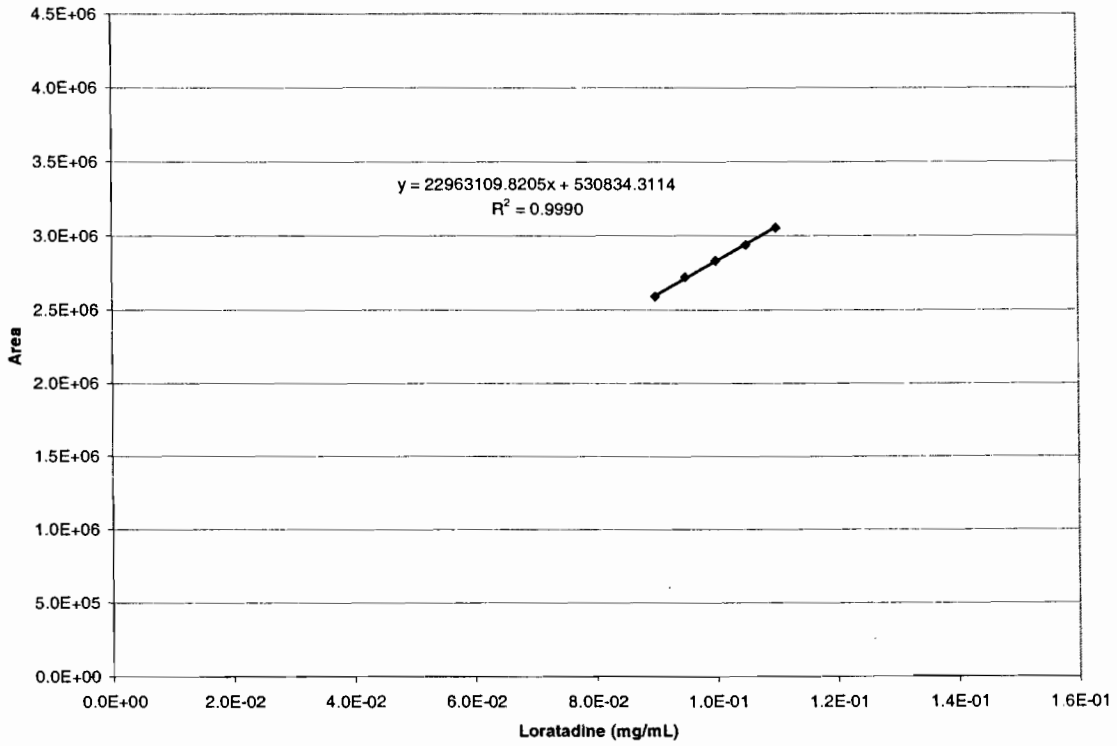


Figure 17: High level linearity for charged aerosol detection validation from 90 to 110% range

For the in-process control assay, the linearity results can be seen in **Figure 18**. The r^2 acceptance criteria was ≥ 0.9500, and the result obtained was 0.9858. Using the low level criteria for this section of the validation this was easily met. However, it is obvious that the range of QL to 50% is not correct for this control method. A more correct linearity result can be seen in **Figure 19**, in which the 50% data point was removed resulting in an r^2 of 0.9974 which would even be acceptable for the high level criteria set for the purity assay.

The term "range" has been mentioned several times in the accuracy and linearity portions of this validation, yet has not been individually discussed. The range of the method is tied very closely into the linearity and accuracy portions of the validation. In order to have a defined range, both the accuracy and the linearity sections must meet their validation criteria, after which the range is subsequently defined. For the method that has been validated thus far, the ranges are different depending on which approach and the type of method. Most notably the high level (purity assay) utilizing either the linear or quadratic curve for the charged aerosol detector. If the purity of the main compound was consistently know to be greater than 95%, then the linear range of 90 to 110% would be adequate for this assay. However, if the purity of the main compound was know to be as low as 89%, then the quadratic range of 50 to 150% would be adequate for this assay. It is not just a matter of which assay is more correct, it is also a knowledge of what the material purity, and whether there enough leeway in the range to deal with potential deviations from the normal.

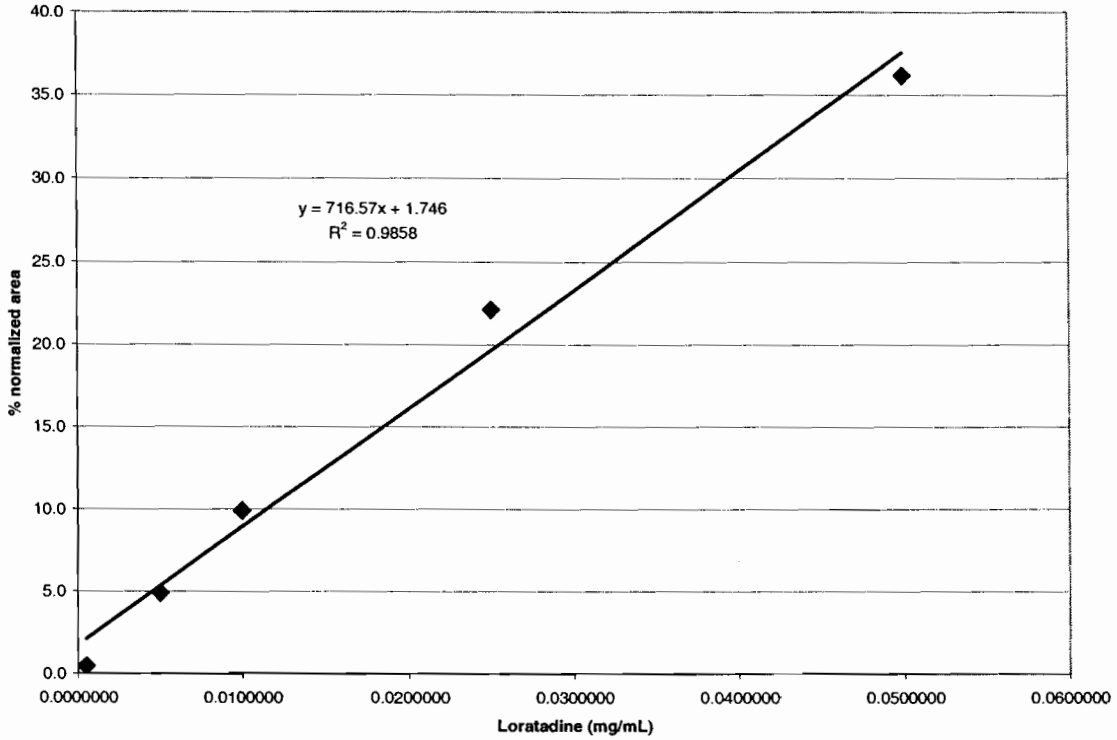


Figure 18: In-process control linearity for charged aerosol detection validation range QL to 50%

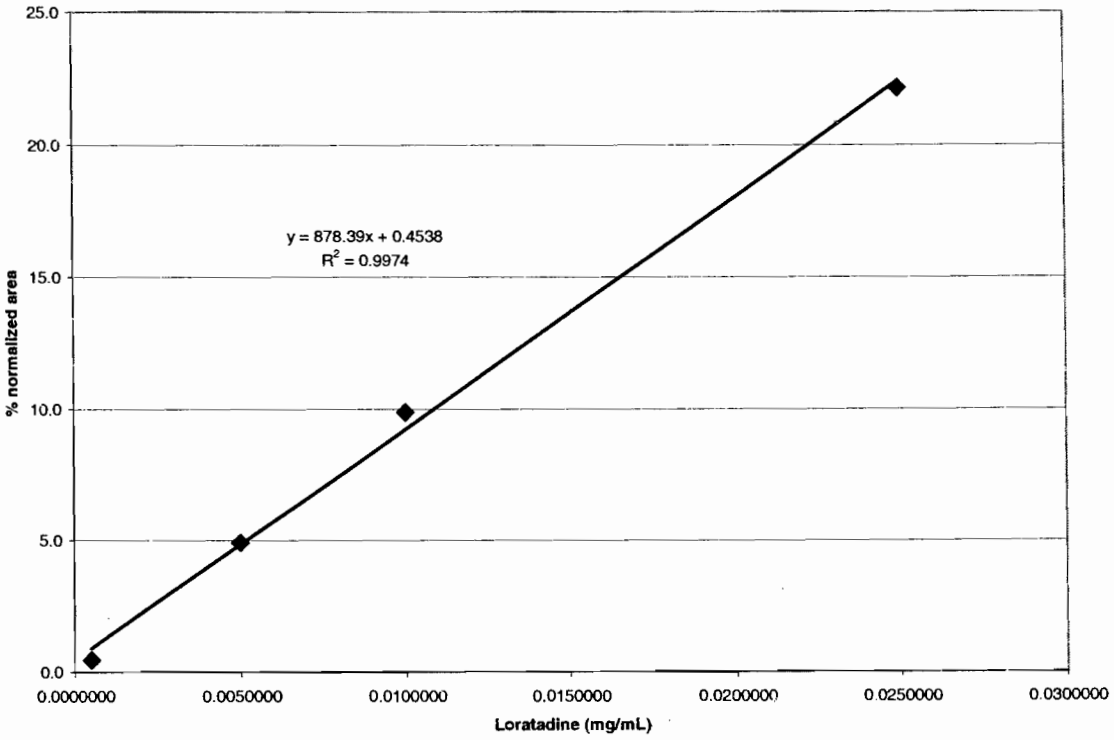


Figure 19: In-process control linearity for charged aerosol detection validation range QL to 25%

4.1.5 Precision and Intermediate Precision

The precision data was generated from the multiple solutions assayed from the accuracy section. The precision data for the charged aerosol detector can be seen in **Table 11**. The results for each different assay type at each different level for the precision section were within the acceptance criteria. The intermediate precision was assayed on the same instrument but a different day with different preparations, and mobile phase. Normally a different system would be used for the intermediate precision, however only one instrument was available for use, so an exception was made. The results for the intermediate precision for the charged aerosol detector can be seen in **Table 12**. The results for each different assay type at each different level for the intermediate precision section were within the acceptance criteria. All of the criteria for accuracy, linearity, and range were also met by the intermediate precision section. This data is typically used for the secondary analyst in the previous sections. The secondary analyst data has been omitted from this document as it would have been repetitive information that would have contributed little to the document. The % RSD for each of the solution levels in the precision section were compared to the % RSD from the intermediate precision section. This result satisfies the last requirement of intermediate precision, that the results from the two different assays must be comparable to each other. These are very valuable results as this section of the validation proves that a HPLC-charged aerosol detection method can be precise and reproducible from assay to assay.

	Solution Level	Precision (%RSD)	Precision Acceptance Criteria
Impurity Assay	0.5%	0.95	± 20.0
	2.5%	0.77	± 20.0
	5.0%	0.81	± 20.0
Purity Assay (Linear)	90.0%	1.61	± 2.0
	100.0%	1.09	± 2.0
	110.0%	0.05	± 2.0
Purity Assay (Quadratic)	50.0%	0.81	± 2.0
	100.0%	1.09	± 2.0
	150.0%	0.45	± 2.0
In-process Control Assay	5.0%	0.95	± 20.0
	10.0%	0.20	± 20.0
	25.0%	0.77	± 20.0

Table 11: Precision data for charged aerosol detection

	Solution Level	Precision (%RSD)	Precision Acceptance Criteria
Impurity Assay	0.5%	1.28	± 20.0
	2.5%	0.74	± 20.0
	5.0%	0.88	± 20.0
Purity Assay (Linear)	90.0%	1.57	± 2.0
	100.0%	1.07	± 2.0
	110.0%	0.02	± 2.0
Purity Assay (Quadratic)	50.0%	0.88	± 2.0
	100.0%	1.07	± 2.0
	150.0%	0.46	± 2.0
In-process Control Assay	5.0%	2.06	± 20.0
	10.0%	1.18	± 20.0
	25.0%	0.26	± 20.0

Table 12: Intermediate Precision data for charged aerosol detection

4.1.6 Solution Stability

Since solution stability is more of an aspect of the method or of the solution, and not as specific to the operating parameters of the charged aerosol detector, a single time point for solution stability was chosen on Day 2 or 48 hours from initial sample injection. The solutions that were examined were the Blank, Quantitation Limit, and the Sample which was the same as the reference material for this validation. System suitability was run followed by an assay of the sample. The solutions were allowed to stand on the laboratory bench top for two days at ambient temperature with no protection from light. Then system suitability was run followed by an assay of the two day old solutions. The results for the blank solution showed that there was no increase or decrease in any peaks. The quantitation limit solution gave a signal to noise ratio of 10:1 at time zero and on day two. The sample solution acceptance criteria was ± 2.0% from the original assayed result. The time zero result for the sample solution was 97.8% and the result for day 2 was 99.1% which is within the acceptance criteria. Therefore all of our solutions for this assay have two day solution stability. The mobile phase also has two day solution stability by passing system suitability on both days and giving acceptable results for the solutions.

4.1.7 Robustness

In order to determine the charged aerosol detector's robustness for an analytical method, deliberately modifications were made, one at a time, to the assay conditions in order to observe the effects. **Table 13** shows the conditions that were modified and to what extent they were modified.

Condition	Modification
Injection Volume	4.5μL
	5.5 μL
Flow Rate	0.9mL/minute
	1.1mL/minute
Gradient Slope	Slope 4
	Slope 8
Column Temperature	35°C
	45°C
Initial % Organic in Mobile Phase	45%
	55%

Table 13: Robustness modifications to method

For the purpose of this validation, the solutions that were assayed for the robustness were the system suitability solutions, Blank, QL, six injections of the Chemical Assay Reference, and in-process control. The results of these solutions and the effects of the robustness changes can be seen in **Table 14.** For all of the blank solutions that were injected, the criteria of acceptance were that there were no peaks in the chromatography that could potentially interfere with the assays. Examining all of the data for the blank solutions, there were no interferences at any of the modified conditions. The quantitation limit solutions that were injected at all of the modified conditions had acceptance criteria of the signal to noise ratio being greater than or equal to 10. All of the quantitation limit solutions had signal to noise ratio greater than or equal to 10. For the chemical assay reference solutions six injections were made at each condition and the areas at each condition needed to have a %RSD less than or equal to 2.0. A overlay of the injections for the chemical assay reference solution for each change in the chromatographic conditions is shown in **Figure 20.** The chemical assay reference solutions all yielded acceptable % RSD of less than or equal to 2.0. The in-process control reference solution needed to meet acceptance criteria for the % normalized area of the reactant peak of 1.0 ± 0.1%, which was met for the solution at each condition. A overlay of the injections for the in-process reference solution for each change in the chromatographic conditions is shown in **Figure 21.**

Condition	Modification	Solution	Result
Initial Conditions	None	Blank	No Interference
		QL	S/N=10
		Chemical Reference	%RSD= 0.7
		In-Process Reference	% Normalized Area=1.0
Injection Volume	4.5μL	Blank	No Interference
		QL	S/N=11
		Chemical Reference	%RSD=1.7
		In-Process Reference	% Normalized Area=0.9
	5.5 μL	Blank	No Interference
		QL	S/N=11
		Chemical Reference	%RSD=2.0
		In-Process Reference	% Normalized Area=1.0
Flow Rate	0.9mL/minute	Blank	No Interference
		QL	S/N=12
		Chemical Reference	%RSD=0.6
		In-Process Reference	% Normalized Area=1.0
	1.1mL/minute	Blank	No Interference
		QL	S/N=10
		Chemical Reference	%RSD=1.7
		In-Process Reference	% Normalized Area=0.9
Gradient Slope	Slope 4	Blank	No Interference
		QL	S/N=21
		Chemical Reference	%RSD=1.1
		In-Process Reference	% Normalized Area=1.0
	Slope 8	Blank	No Interference
		QL	S/N=11
		Chemical Reference	%RSD=1.1
		In-Process Reference	% Normalized Area=0.9
Column Temperature	35°C	Blank	No Interference
		QL	S/N=11
		Chemical Reference	%RSD=0.7
		In-Process Reference	% Normalized Area=0.9
	45°C	Blank	No Interference
		QL	S/N=10
		Chemical Reference	%RSD=1.8
		In-Process Reference	% Normalized Area=0.9
Initial % Organic in Mobile Phase	45%	Blank	No Interference
		QL	S/N=14
		Chemical Reference	%RSD=2.0
		In-Process Reference	% Normalized Area=1.0
	55%	Blank	No Interference
		QL	S/N=11
		Chemical Reference	%RSD=0.3
		In-Process Reference	% Normalized Area=0.9

Table 14: Results for Robustness

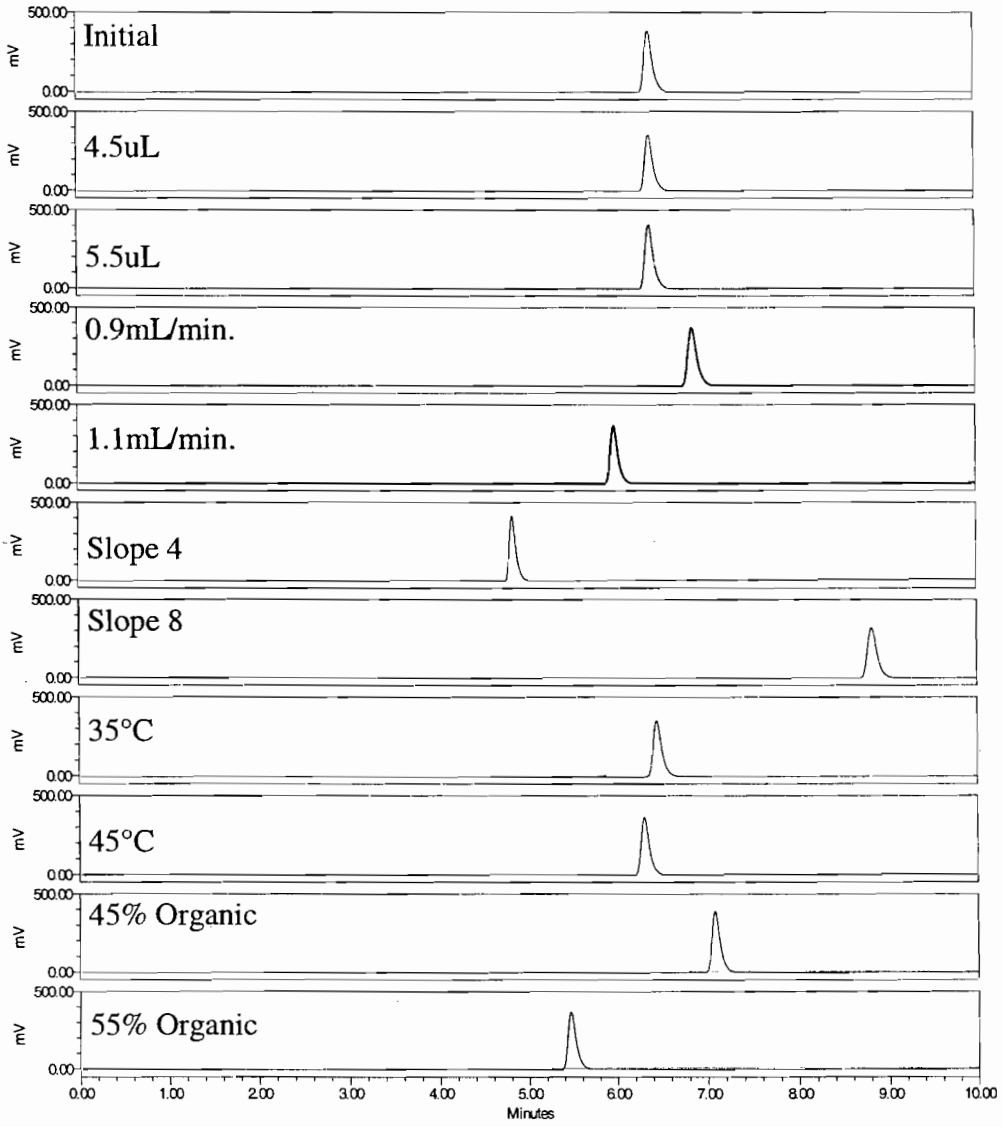


Figure 20: Robustness Chromatography of chemical assay reference solution

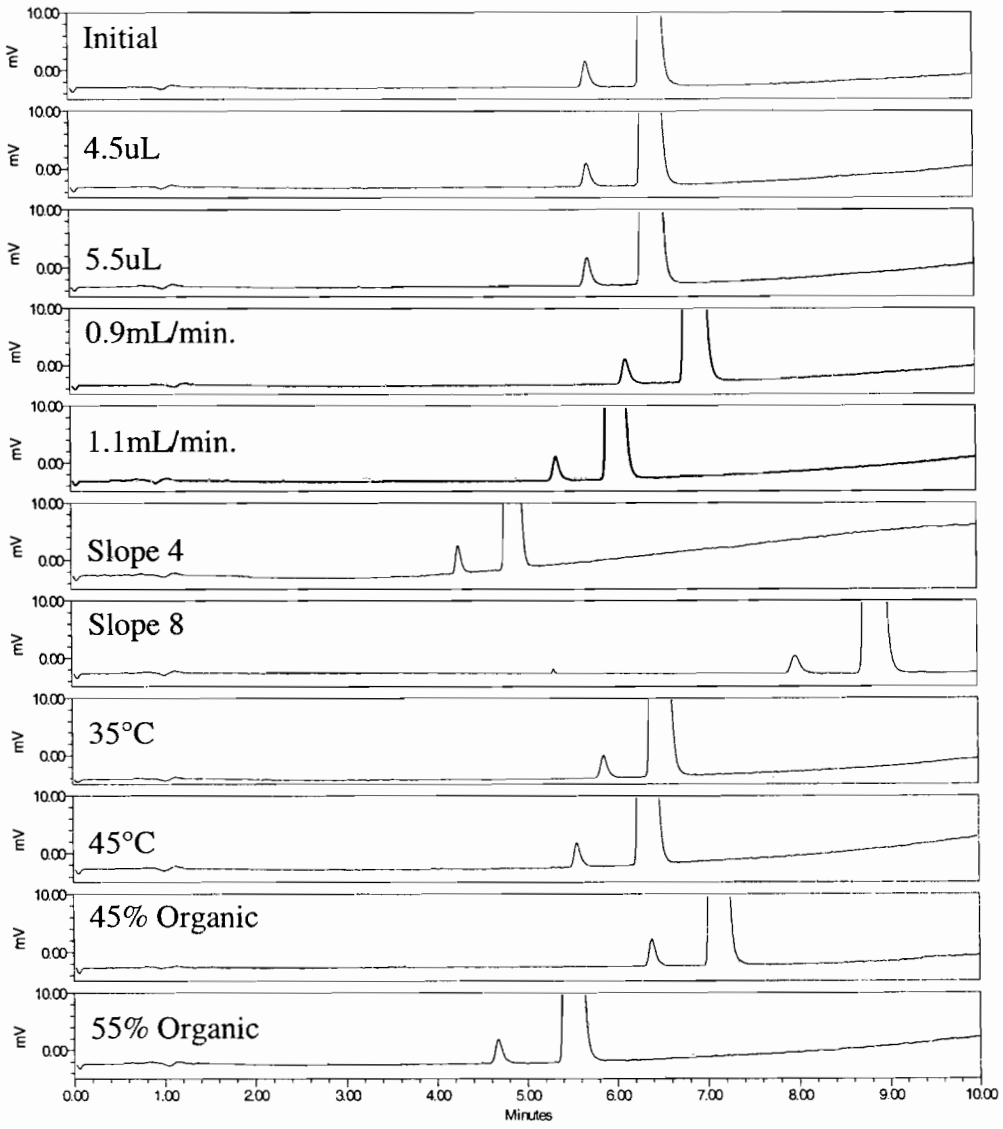


Figure 21: Robustness Chromatography of in-process control solution

Conclusion

The validatability of a HPLC method using charged aerosol detection was examined for several different types of assays (impurity, purity, and in-process) using a hypothetical process. It was determined that these methods were fully validatable using slightly modified criteria from those typically used for HPLC method with UV detection. When deciding on which criteria to set for the charged aerosol detector, the standard validation criteria are an acceptable place to begin. However, in some cases the criteria may not be acceptable for a non-linear for of detection with a HPLC-charged aerosol detection assay. This requires good scientific judgment when drafting the validation plan. Acceptance criteria can be altered, but only to the point where the validation still proves that there are good controls in the method and that it is accurate, precise, reproducible, and robust over the range that is being utilized. By having successfully undergone pharmaceutical validation - one of the most difficult test for any analytical technique - the charged aerosol detector has been shown to be a useful and valuable detector for use with high performance liquid chromatography.

4.2 Effects of HPLC Parameters on Charged Aerosol Detection

4.2.1 Charged Aerosol Detection Mobile Phase Background

The purpose of this experiment was to gain an understanding of how the mobile phase alone might contribute to the analytical response, if at all. The column was replaced with a length of PEEK tubing for to yield a sufficient back pressure. The purpose of not having a column in this experiment is to remove any potential mobile phase/stationary phase interactions that might affect the response seen by the detector. A column could potentially add other response altering affects to our experiment in the form of retained compounds eluting from the column, and bonded phase being removed from the silica. These problems are generally not issues with other traditional detection techniques, such as UV, as they will not typically see the bonded phase. This could be an issue with charged aerosol detection due to its ability to detect non-chromophoric compounds. The use of the gradient proportioning valve (GPV) for the mixing of the mobile phases will have give slightly different ratios of water to acetonitrile and water to methanol rather than if each of the two components were premixed and run by a isocratic method but was ignored for this study as the overall trend is still observable.

Figure 22 shows the chromatographic results obtained for the change from 100% water to 100% methanol by changes to the composition of the mobile phase made by the gradient proportioning valve over the course of the run.

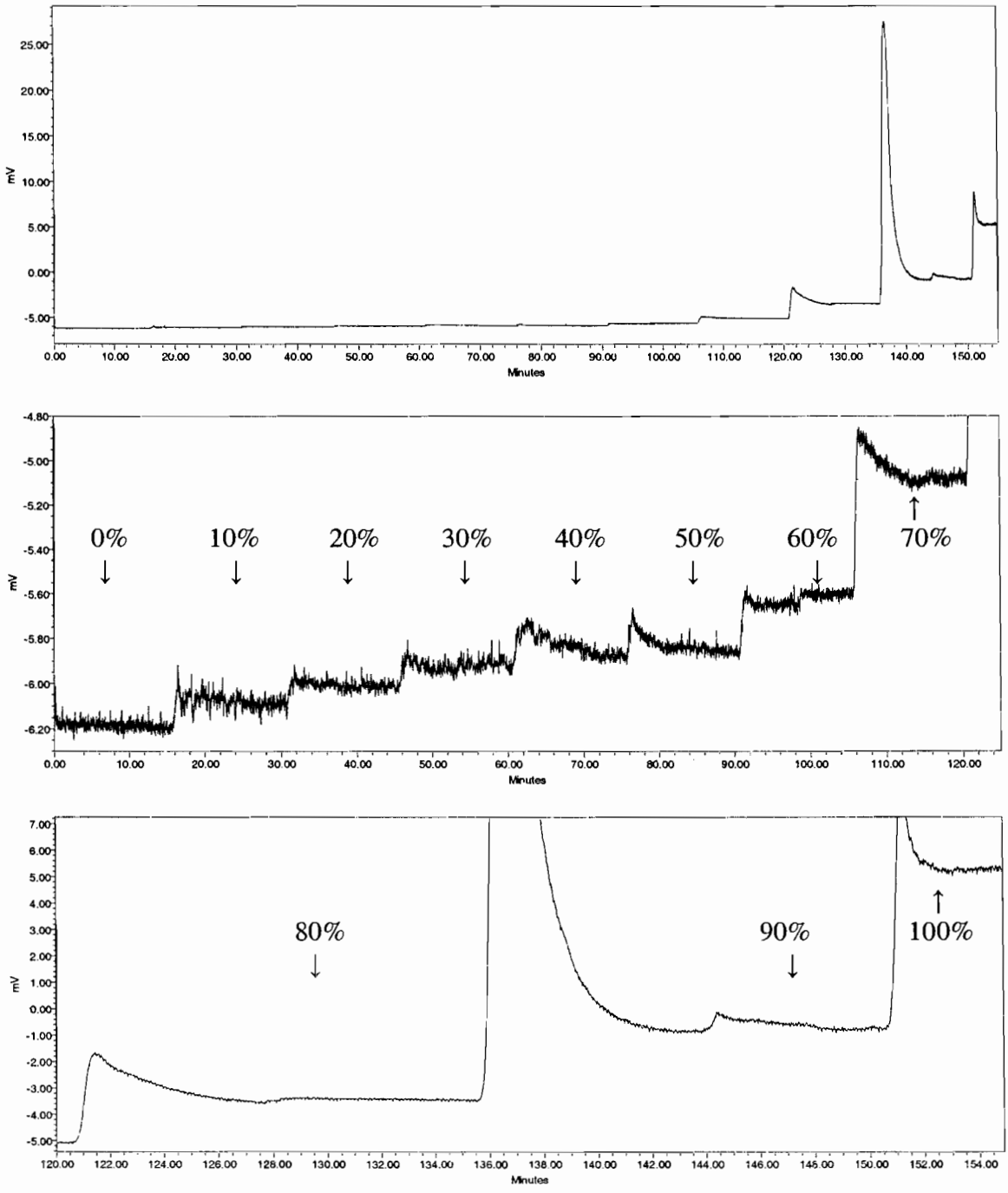


Figure 22: Chromatogram of water versus methanol in various mixtures full scale(top) and zoomed (bottom)steps of 10%.

The main focus of this experiment was to observe a general trend when the mobile phase composition is altered; the slight difference given by the GPV is not a concern for the purpose of this experiment.

The top chromatogram is the full scale results for the entire run. For the mobile phase composition of water:methanol at 10:90 v/v a drastic rise in signal is observed as compared to the other changes to the mobile phase. This type of drastic increase in response and subsequent return to a lower baseline response has been observed for some drastic instantaneous changes. The middle chromatogram is the magnified version of the top chromatogram with a focus on the response of the mobile phase from the composition at 100% water to water:methanol (30:70) v/v. The bottom chromatogram is the magnified version of the top chromatogram with a focus on the response of the mobile phase from the composition at water:methanol (20:80) v/v to 100% methanol.

Figure 23 is the chromatographic result obtained for the change from 100% water to 100% acetonitrile by isocratic changes to the composition of the mobile phase by steps of 10%. The chromatogram is the full scale results for the entire run. **Table 15** shows the baseline response in mV observed for the different water:methanol and water:acetonitrile mobile phases. The data obtained for this was then plotted and can be seen in **Figure 24**. Comparing the methanol data to the acetonitrile for initial signal at the 100% water the points do not match up even though both should give the same exact response. There are explainable

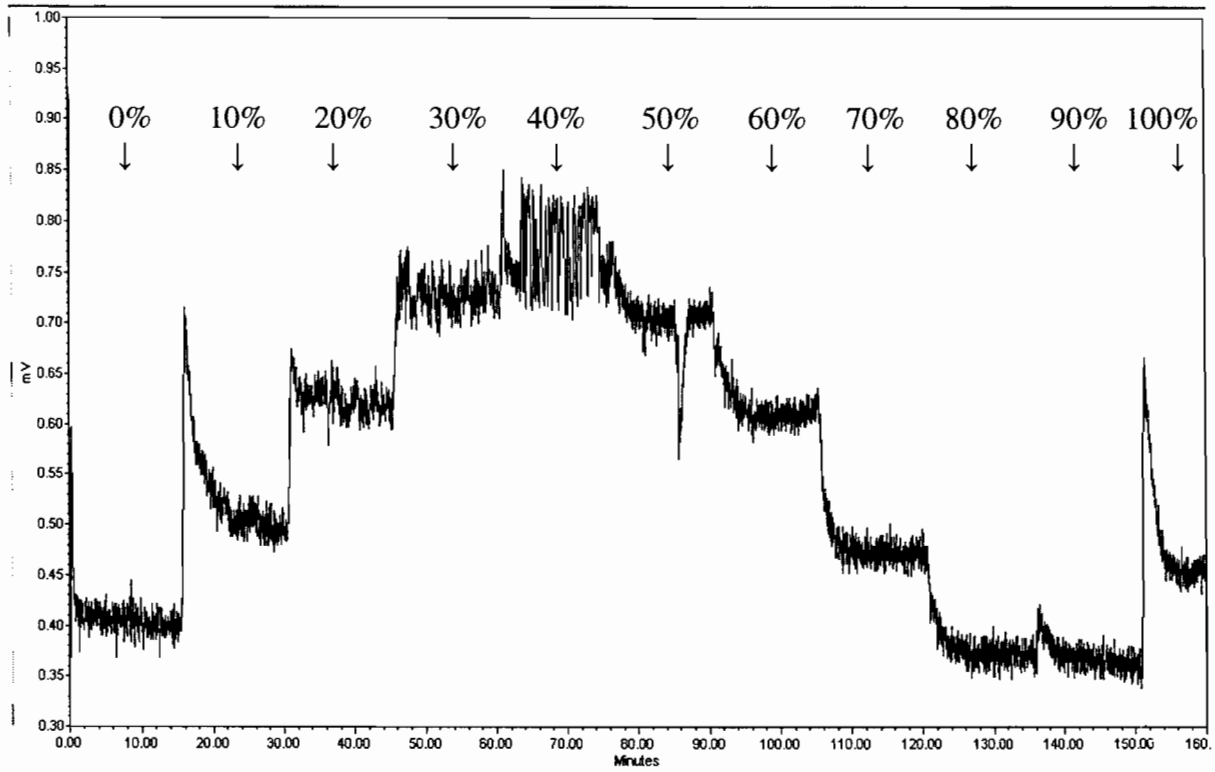


Figure 23: Chromatogram of water versus acetonitrile changes full scale(top) and zoomed (bottom)

Mobile Phase (Water:Organic) %v/v	Baseline Response for Methanol (mV)	Baseline Response for Acetonitrile (mV)
100:0	-6.17	0.40
90:10	-6.09	0.48
80:20	-5.99	0.62
70:30	-5.92	0.72
60:40	-5.90	0.76
50:50	-5.86	0.71
40:60	-5.60	0.62
30:70	-5.10	0.48
20:80	-3.40	0.37
10:90	-0.60	0.36
0:100	5.40	0.46

Table 15: Response (mV) of charged aerosol detection with respect to changes in mobile phase composition

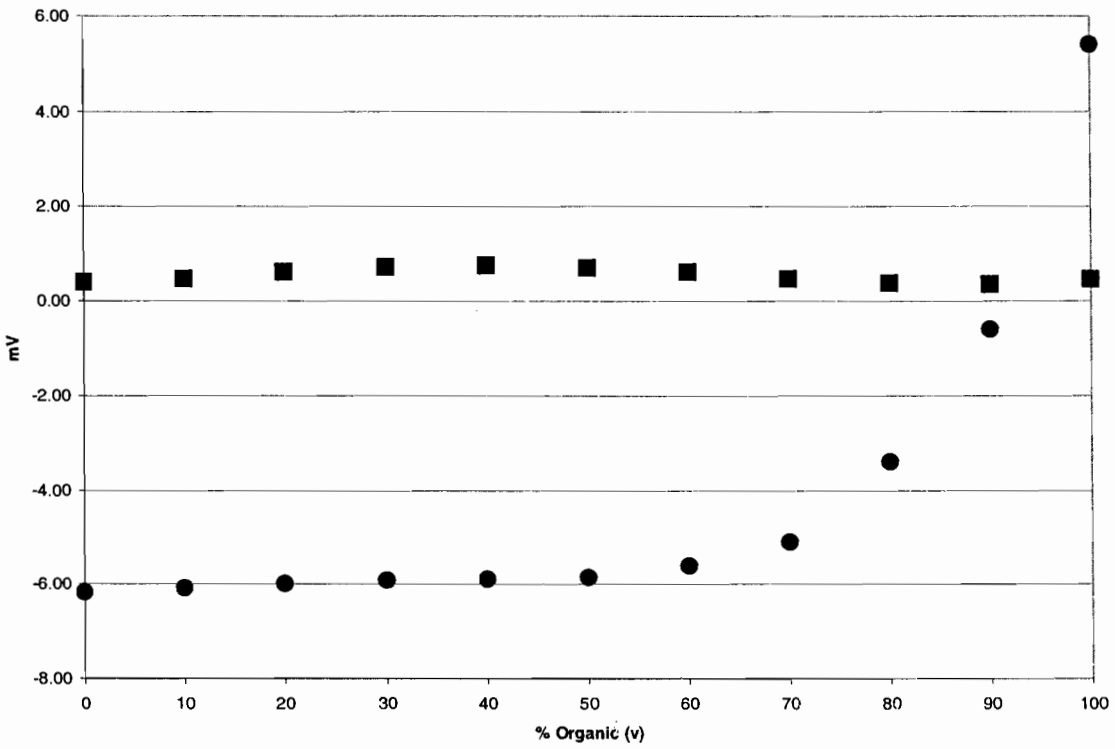


Figure 24: Response (mV) of charged aerosol detection with respect to changes in mobile phase composition (Methanol • ; Acetonitrile ■)

reasons for this discrepancy; the data for these experiments were done many days apart that can have been a factor seen as a slight detector drift, of the purity/pH of the water was different from bottle to bottle is the most likely reason. This detector has an auto-zero function that can be setup to zero upon injection from the HPLC, however this function was not used in any of the experiments. The highly sensitive charged aerosol detector can detect even the slightest differences from one mobile phase to another, such as in the data seen for 100% water. The detector was therefore zeroed before each use with no mobile phase flow. This was done to have a consistent baseline for the research and is a typical practice when utilizing the charged aerosol detector. By zeroing the detector with no flow and only the nitrogen gas flowing, determining the mobile phase consistency from preparation to preparation is possible. For the same mobile phase with multiple preparations, differences that would not be detectable on a UV detector may be seen by charged aerosol detection and can give valuable information for consistency from one assay to the next. For example if there was a salt contaminant in the mobile phase and the separation was a typical reverse phase system where the salt would elute from the column at a constant rate a UV/Vis detector would not be effected. However, a charged aerosol detector would register this constant elution of salt as an increase in the over all baseline and potentially an increase in the noise in the baseline.

Examining the data that was obtained for the water to methanol changes in the baseline for the charged aerosol detector, there is an increase in signal output by

the detector as the mobile phase composition moves from 100% water to 100% methanol. This increase over the range from 0% methanol to 70% methanol shows a change in response of the baseline of 1.07 mV. The maximum output for the detector at the 100 pA range setting is approximately 1000 mV, after which the signal has reached a maximum. Therefore over this range a potential response discrepancy due to the baseline interference would be 0.107%. Increasing to 80% methanol this potential response discrepancy due to the baseline interference would be 0.277%, at 90% methanol 0.557%, and at 100% methanol 1.157%.

Examining the data that was obtained for the water to acetonitrile changes in the baseline for the charged aerosol detector, there is an increase in signal output by the detector as the mobile phase composition moves from 100% water to 100% acetonitrile. This increase over the range from 0% acetonitrile to 40% acetonitrile shows a change in response of the baseline of 0.36mV. Therefore over this range a potential response discrepancy due to the baseline interference would be 0.036%. This is the maximum deviation from the initial conditions observed over the entire acetonitrile range. In addition charged aerosol detection shows an increase in baseline nose at 40% acetonitrile as compared to all of the other mobile phase concentrations.

Comparing the data obtained for methanol and acetonitrile, the acetonitrile would be more favorable over the entire concentration range for its minimal effect on

the electrometer if an assay were running a gradient. Methanol can be use in a gradient over the entire concentration range, however there will be more of a baseline rise towards the end of the gradient. Any of the mobile phases that have been examined here would be acceptable for use in isocratic mode. A comparison of charged aerosol detection to other detection techniques, such as UV-Vis or ELSD, the baseline rise due to mobile phase for the charged aerosol detector is remarkably stable under any of the mobile phase conditions examined here. Possible reasons for the slight baseline changes could be accounted by sheering effects upon nebulization resulting in different droplet sizes or a different spray area pattern, viscosity differences resulting in a change in the pump performance. The mobile phase compositions examined in this section had very little effect on the baseline signal output from the charged aerosol detector. Therefore no further examination was performed in order to determine the reason behind the very slight variation.

4.2.2 Determination of Mobile Phase Associated with Charging

In **Table 16** the results for the volume of waste bottle liquid that was collected from the varied mobile phase concentrations at 1.0mL/minute for 100 minutes. If none of the mobile phase was nebulized and it all drained into the waste bottle an expected value of 100mL should have been collected in the waste bottle. If half of the mobile phase was nebulized and continued on to the electrometer an expected value of 50mL should have been collected in the waste bottle. Examining **Figure 25** the volume collected was dependent upon the composition

%v/v Water	%v/v Organic	Methanol Total (mL)	Acetonitrile Total (mL)
100	0	94.0	94.0
90	10	93.0	88.5
80	20	91.0	84.5
70	30	89.0	79.0
60	40	87.5	75.0
50	50	85.0	69.0
40	60	82.0	69.0
30	70	77.5	70.0
20	80	73.0	70.0
10	90	68.0	70.0
0	100	62.0	65.0

Table 16: Amount for organic:water waste bottle recovered mobile phase

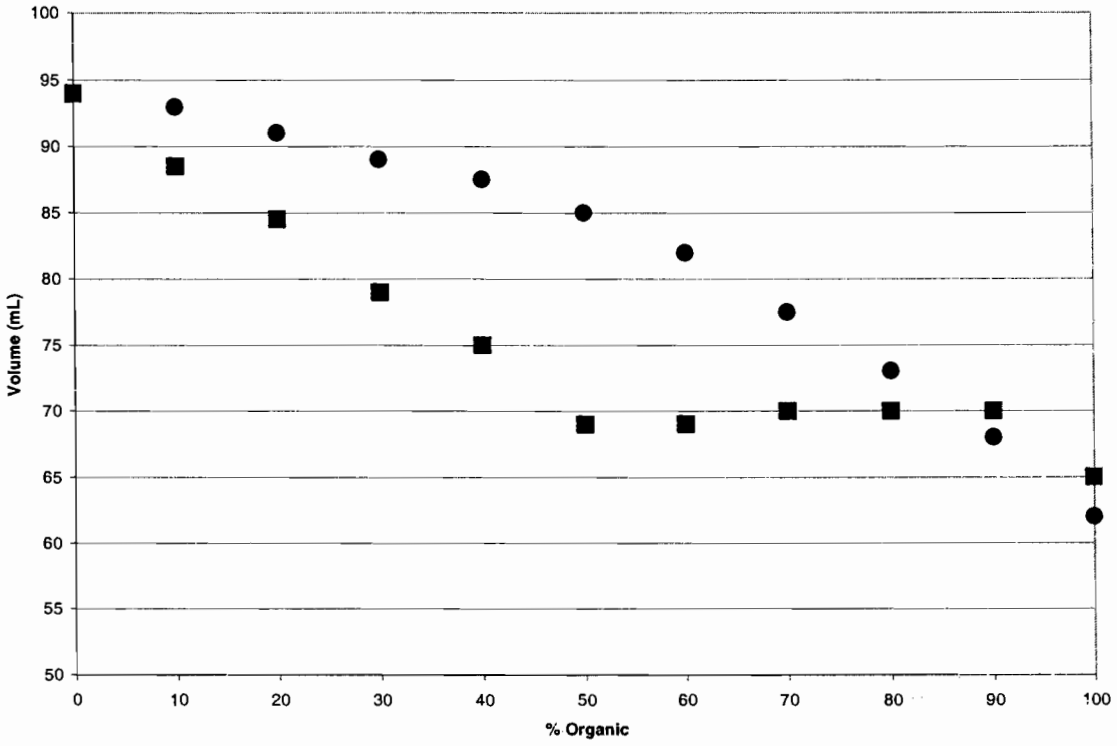


Figure 25 : Amount for organic:water waste bottle recovered mobile phase (Methanol • ; Acetonitrile ■)

of the mobile phase. Methanol and acetonitrile showed a similar characteristic that as their concentrations increased and the water concentration decreased in the mobile phase the amount of mobile phase that was collected in the waste bottle decreased.

Another way of saying this would be that as the methanol or acetonitrile concentrations in the mobile phase increased and the water concentration decreased in the mobile phase the amount of mobile phase that moves past the spray chamber increased. Notable in the plot of this data would be that acetonitrile has a region from 50% to 90% where the volumes are rather stable. This data on the volume of mobile phase that goes to waste versus the volume that is aerosol and continued on past the spray chamber is useful, but volumes alone can not be used to determine the composition of the waste collected, water to methanol or acetonitrile. In order to determine the make up of the aerosol that continued on past the spray chamber the waste that was collected was examined. **Table 17** shows the first property of the waste bottle solutions that was examined, the density. As expected the density of the collected waste was around 1.00g/mL when the composition of the mobile phase was 100% water, and decreased in density as either methanol or acetonitrile was added. A similar trend was observed for the densities of the un-nebulized mobile phases themselves. Comparing the un-nebulized mobile phases to the volumes collected for the waste, a discrepancy in the densities at each mixture level is observed. This means that there is some difference in the composition of the

Methanol:Water Mobile Phase

%v/v Water	%v/v MeOH	Density of Mobile Phase (g/mL)	Density of Waste (g/mL)	Total Amount of Waste (g)
100	0	0.9934	0.9963	93.7
90	10	0.9827	0.9847	91.6
80	20	0.9678	0.9734	88.6
70	30	0.9550	0.9618	85.6
60	40	0.9408	0.9490	83.0
50	50	0.9229	0.9329	79.3
40	60	0.9010	0.9145	75.0
30	70	0.8773	0.8919	69.1
20	80	0.8490	0.8646	63.1
10	90	0.8168	0.8297	56.4
0	100	0.7850	0.7841	48.6

Acetonitrile:Water Mobile Phase

%v/v Water	%v/v MeCN	Density of Mobile Phase (g/mL)	Density of Waste (g/mL)	Total Amount of Waste (g)
100	0	0.9979	0.9968	93.7
90	10	0.9850	0.9924	87.8
80	20	0.9689	0.9817	83.0
70	30	0.9490	0.9732	76.9
60	40	0.9277	0.9602	72.0
50	50	0.9029	0.9446	65.2
40	60	0.8820	0.9078	62.6
30	70	0.8562	0.8618	60.3
20	80	0.8308	0.8287	58.0
10	90	0.8027	0.8023	56.2
0	100	0.7775	0.7735	50.3

Table 17: Density and calculated weight results for mobile phases

volumes collected from waste as compared to the original un-nebulized mobile phase. Combining the data from the volume of waste collected with the data collected for the densities of the waste collected, it is possible to determine the total weight of the waste collected.

Waste (mL) **x** Density (g/mL) = Total Waste (g) (3)

In order to determine the amount of methanol or acetonitrile that is actually contained in this volume of waste that was collected another technique was utilized, Karl Fischer Titration. This technique is actually a titration for water, and not methanol or acetonitrile. Since all of the mobile phases that only have two components, one of them being water, whatever the amount of water is determined in the waste volumes the rest must be the other component, either methanol or acetonitrile. In **Table 18 & 19** the results from the Karl Fischer Titrations and the calculated percent organic can be seen. The data trend that can be observed for both methanol and acetonitrile mobile phases is that as the amount of organic solvent decreases the amount of water increases, and this is obvious result. The volumes of waste that were collected show a similar trend to the mobile phases themselves. However, it is notable that for all of the mixtures of mobile phases, the corresponding collected waste has an increased percentage of water.

%v/v Water	%v/v MeOH	%w/w Water in Mobile Phase	%w/w Water in Waste
100	0	100	100
90	10	92	93
80	20	83	87
70	30	75	80
60	40	65	67
50	50	56	59
40	60	45	50
30	70	35	41
20	80	24	29
10	90	12	15
0	100	0	0

%v/v Water	%v/v MeCN	%w/w Water in Mobile Phase	%w/w Water in Waste
100	0	100	100
90	10	92	98
80	20	83	93
70	30	75	89
60	40	66	80
50	50	56	74
40	60	46	59
30	70	36	45
20	80	24	30
10	90	13	14
0	100	0	0

Table 18: Karl Fisher results for organic:water mobile phase

%v/v Water	%v/v MeOH	%w/w MeOH in Mobile Phase	%w/w MeOH in Waste
100	0	1	0
90	10	8	8
80	20	17	13
70	30	25	20
60	40	35	33
50	50	44	41
40	60	55	50
30	70	65	59
20	80	77	72
10	90	88	85
0	100	100	100

%v/v Water	%v/v MeCN	%w/w MeCN in Mobile Phase	%w/w MeCN in Waste
100	0	1	0
90	10	8	2
80	20	17	7
70	30	25	11
60	40	34	20
50	50	44	26
40	60	54	41
30	70	65	55
20	80	76	70
10	90	87	87
0	100	100	100

Table 19: Calculated %w/w organic results for organic:water mobile phase

Water in Waste (g)=

$$\frac{\text{Waste (mL)} \times \text{Density (g/mL)} \times \text{Water (\%w/w)}}{100} \quad (4)$$

Organic Solvent in Waste (g)=

$$\frac{\text{Waste (mL)} \times \text{Density (g/mL)} \times \{100 - \text{Water (\%w/w)}\}}{100} \quad (5)$$

Combining all of the data acquired thus far it is possible to calculate the amount of methanol or acetonitrile that continues on past the spray chamber towards the electrometer.

Organic Solvent Continuing towards Electrometer (g)=

Organic Solvent in Mobile Phase (g) – Organic Solvent in Waste (g) (6)

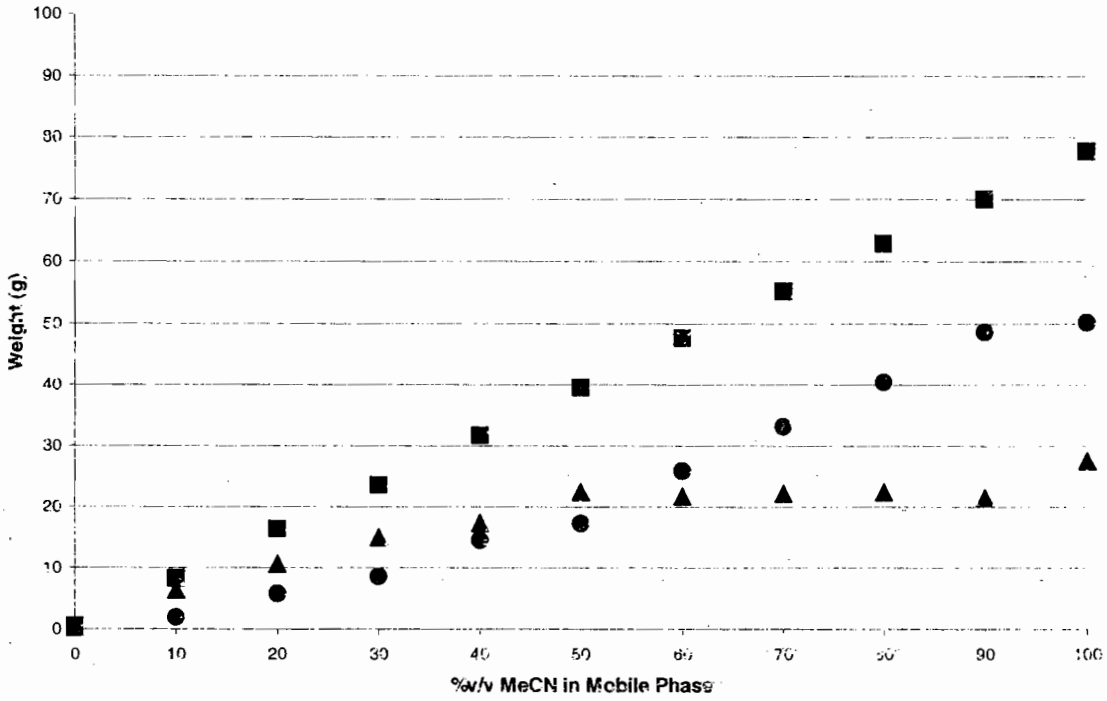
The calculated results for the amount (g) of organic solvent (methanol or acetonitrile) continuing towards the electrometer can be found in **Table 20**. Plotting this data, which can be seen in **Figures 26,** reveals a distinct but very different profile for the two organic solvents that are being examined with charged aerosol detection. The methanol shows a relatively flat response from 20% to 50%. This may suggest a region of signal stability, where despite changes in the mobile phase composition the effect on an analyte might be minimized. The rest of the profile for methanol from 50% to 100% has an increasing trend, which suggests that there can be some more drastic changes of

Mobile Phase		Mobile Phase into Charged Aerosol Detector		Amount going towards Electrometer	
%v/v Water	%v/v MeOH	Water (g)	MeOH (g)	Water (g)	MeOH (g)
100	0	98.2	1.1	4	1
90	10	90.2	8.1	5	1
80	20	80.2	16.5	3	5
70	30	71.3	24.2	3	7
60	40	61.1	33.0	5	6
50	50	51.3	41.0	5	8
40	60	40.8	49.3	4	11
30	70	30.4	57.3	2	16
20	80	20.0	64.9	2	20
10	90	9.9	71.7	1	24
0	100	0.0	78.5	0	30

Mobile Phase		Mobile Phase into Charged Aerosol Detector		Amount going towards Electrometer	
%v/v Water	%v/v MeCN	Water (g)	MeCN (g)	Water (g)	MeCN (g)
100	0	99.3	0.5	6	0
90	10	90.2	8.3	4	6
80	20	80.6	16.3	3	11
70	30	71.4	23.5	3	15
60	40	61.1	31.7	3	17
50	50	50.7	39.5	3	22
40	60	40.6	47.6	4	22
30	70	30.4	55.2	3	22
20	80	20.3	62.8	3	22
10	90	10.2	70.1	3	21
0	100	0.0	77.7	0	26

Table 20: Calculated weight amounts of organic portion of mobile phase going towards electrometer for organic:water mobile phase

Changing mobile phase composition and the quantity of acetonitrile



Changing mobile phase composition and the quantity of methanol

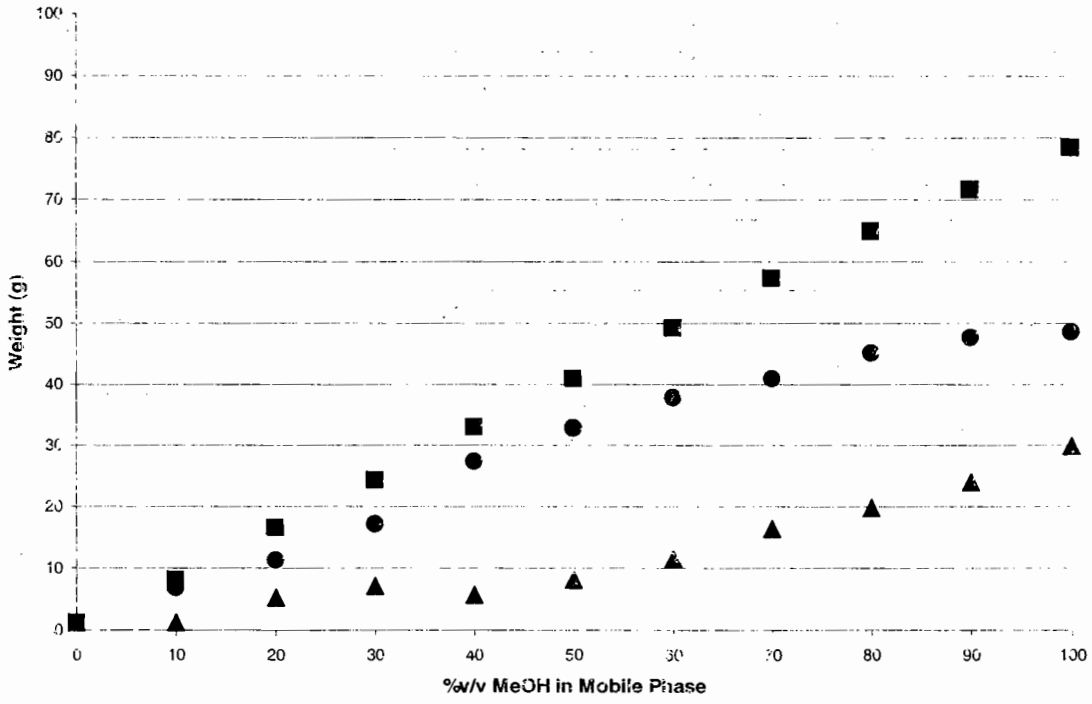


Figure 26 : Changing mobile phase composition and the quantity of organic in mobile phase (■); in waste (●); going towards electrometer (▲)

signal from an analyte due to the mobile phase composition. The acetonitrile shows a relative flat region around 50% to 90%. This may suggest a region of signal stability similar to the region seen in the methanol profile. The rest of the profile for acetonitrile from 0% to 40% has an increasing trend.

4.2.3 Response of Analyte by HPLC-Charged Aerosol Detection

Up to this point in the study of the mechanisms of charged aerosol detection there have been no injections of analyte of any kind. This was deliberate; a basic foundation of how the mobile phase was behaving was first laid. Understanding the amount and composition of the mobile phase that went to waste and that continued on to the detector needed to be understood before examining how these differences might have an effect on the analyte injected onto the system and ultimately detected by the charged aerosol detector.

Loratadine was selected for this response study. A simple gradient HPLC method was developed for this separation. The concentration of the analyte was varied over a wide range in order to determine the charged aerosol detector profile If the charged aerosol detector is linear then it would be similar to UV detectors. If the charged aerosol detector has multiple different curved regions then it would be similar to evaporative light scattering detection (ELSD). Testing these hypotheses would determine how analytes respond in charged aerosol detection. The overlaid chromatograms of the different concentrations of Loratadine injected and separated by this HPLC method and detected by

charged aerosol detection can be seen in **Figure 27.** At first glance this seems typical of most UV detector responses, as the concentration increases, the height and area of the peak increase in a linear fashion.

This initial observation of the charged aerosol detector's response for an analyte as a linear trend based on the overlay of the chromatography is incorrect. By plotting the areas from the injections of Loratadine, a different profile that is non-linear is observed, see **Figure 28**. In the upper plot a best fit linear curve was fitted onto the data. The r^2 is giving a value of 0.9922, however by simple observation this is not a random deviation of the data from the best fit line, and it is simply the incorrect curve. In the bottom plot a quadratic curve was fitted onto the data. The r^2 is 0.9998, and the fit clearly shows that the quadratic curve is a better description of the loratadine response. It would be possible to use a linear model for quantitation with charged aerosol detection with well defined limitations. The limitations that would have to be accepted would be the range. As with any curve, even a circle, taking smaller and small regions of that curve, eventually it will become a near linear curve. This holds true with charged aerosol detection, since the curvature of the Loratadine curve is not as dramatic as the curvature of a circle, rather large regions can be used with a forced linear distribution with acceptable results for quantitation.

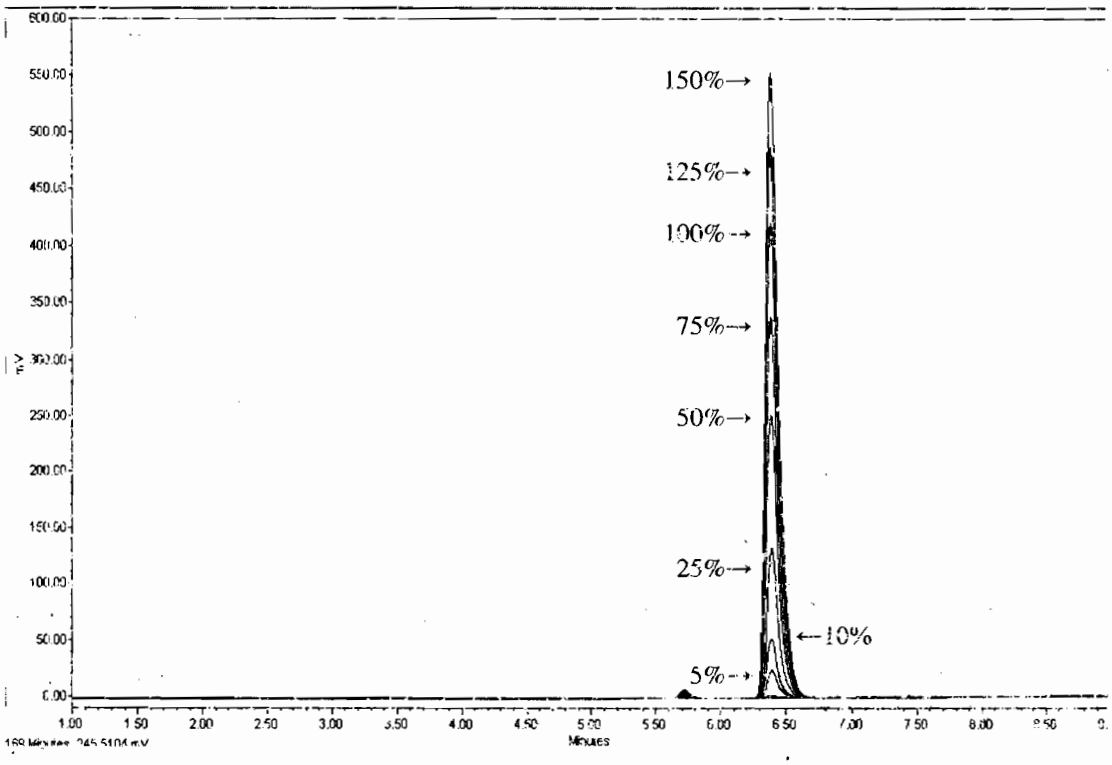
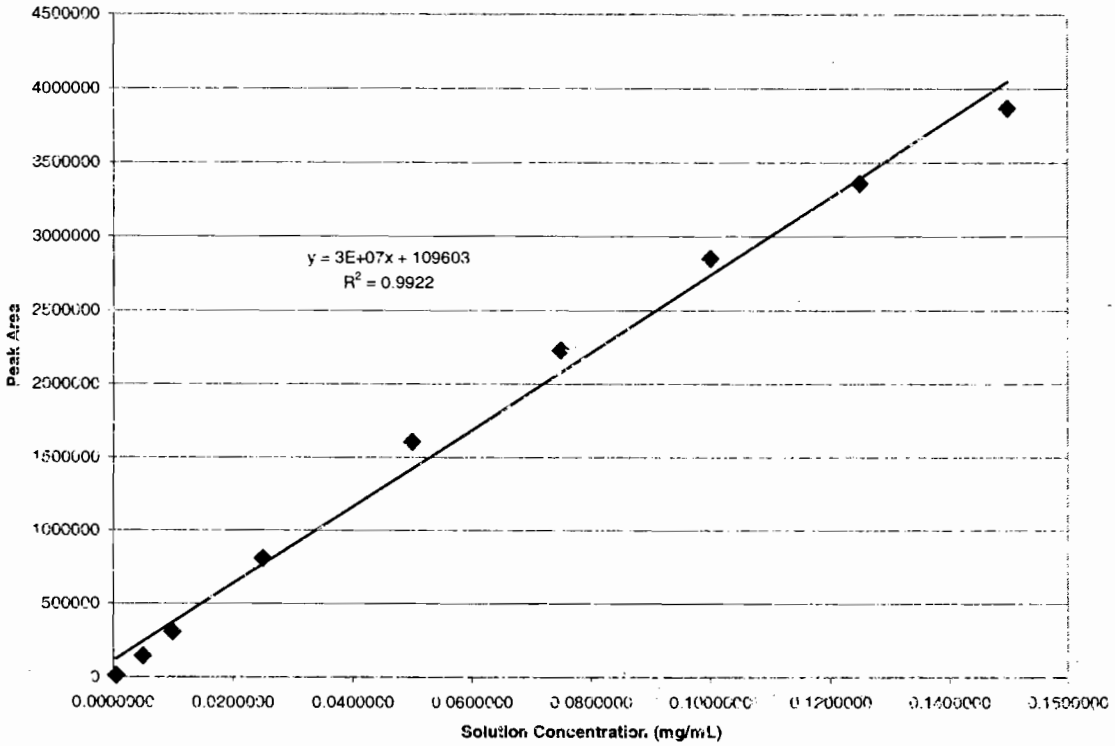


Figure 27: Overlay of injections of Loratadine at varying concentrations, the quantitation limit is barely visible on the scale above.



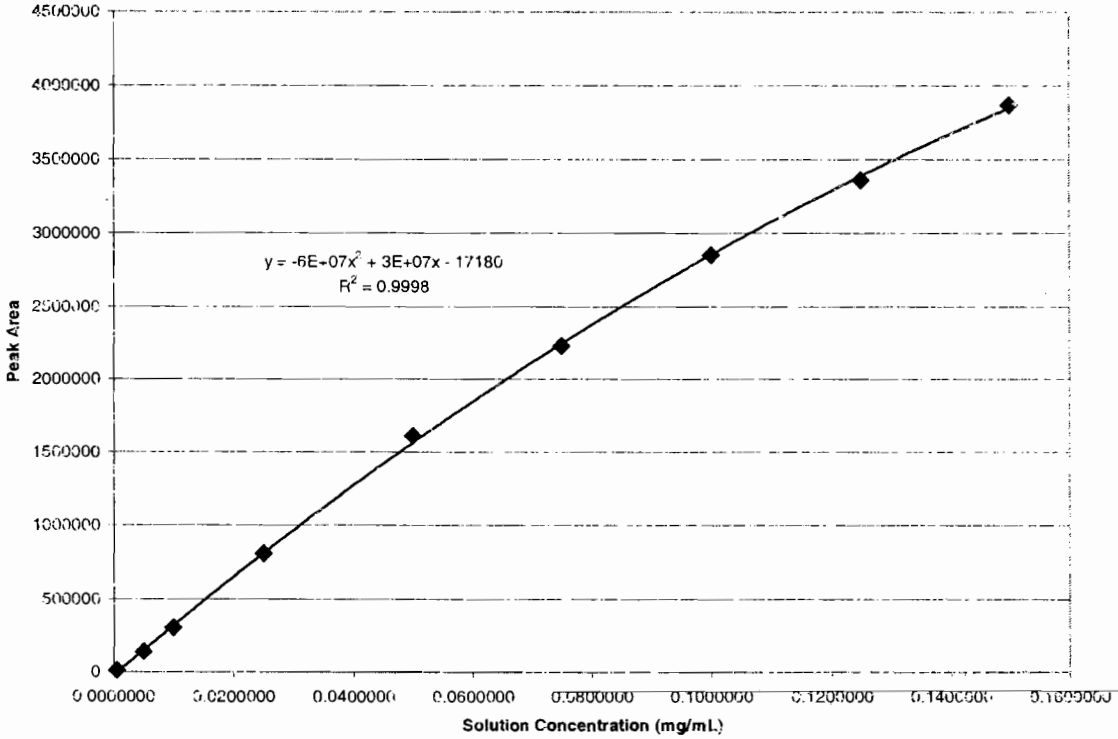


Figure 28: Area plot of loratadine at varying concentrations (top) best fit linear ; (bottom) best fit quadratic

The quadratic nature of the charged aerosol detector response is an important aspect of the detector that needed to be established before moving on to more complex concepts such as the effect of the mobile phase composition on an analyte that has been injected onto the system. For this study there were two different concentrations that were injected onto the system. These were constant while the concentration of the mobile phase was the variable. In addition to varying the mobile phase the diluent was also varied accordingly. This was done in order to eliminate any additional effects that may occur with partial diffusion of diluent injected on to the HPLC and that effecting longitudinal diffusion or even nebulization. If the diluent has the same exact composition as the mobile phase, there is no potential for any of these additional effects.

Figure 29 shows the results of loratadine, albuterol, and mometasone furoate injected at a constant volume and concentration with the only variable being the comporsition of the diluent and mobile phase. Albuterol and mometasone furoate at both concentrations injected for both methanol and acetonitrile show matching response at each mobile phase composition change. Albuterol was only examined in methanol due to its poor solubility in acetonitrile. While the profile for the response for albuterol matched the other two API's, it did not overlay exactly as did loratadine and mometasone furoate. Loratadine and mometasone furoate did deviate in response towards higher and higher

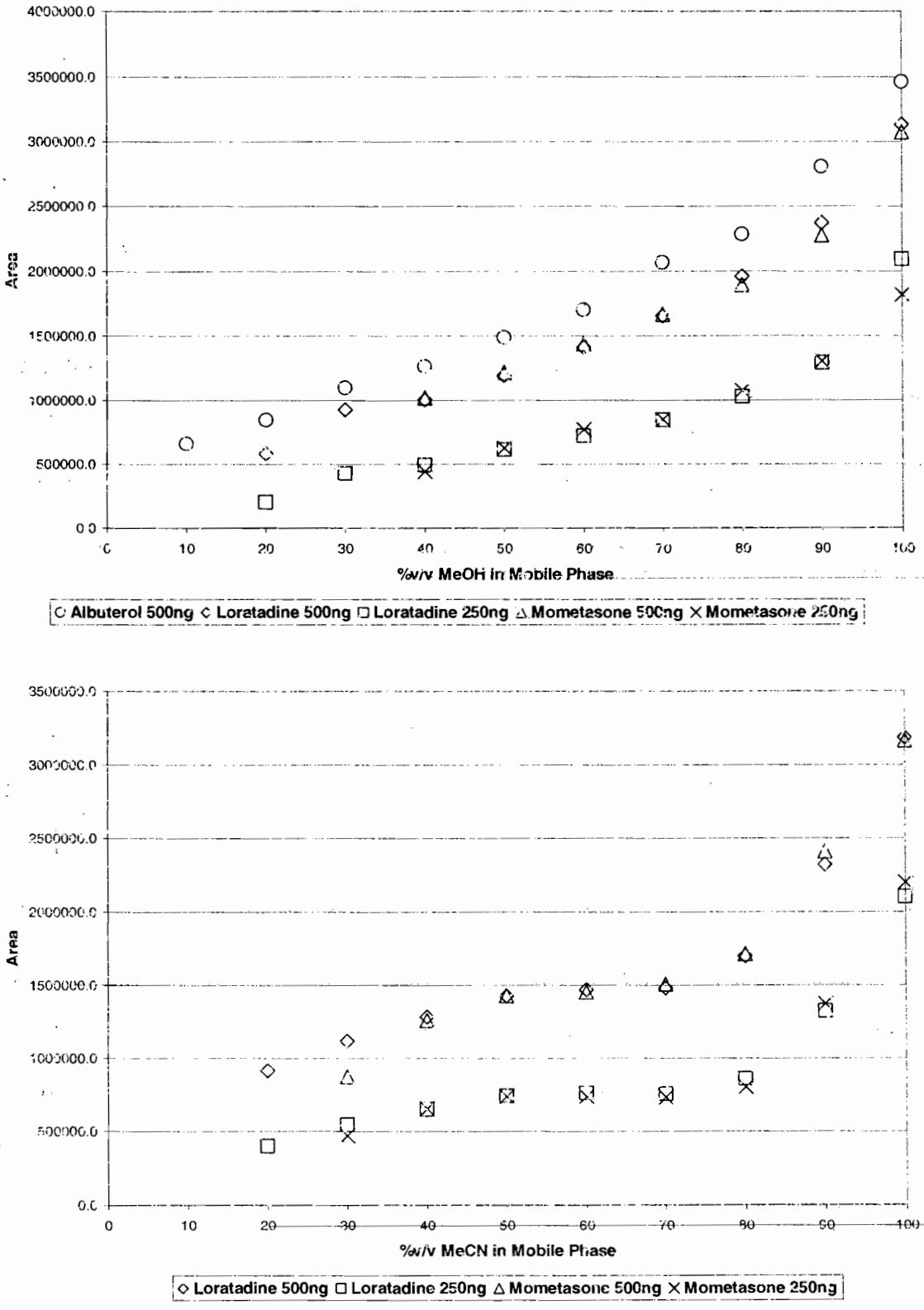


Figure 29: Area of API's versus composition of organic in mobile phase (top) methanol (bottom) acetonitrile.

concentrations of water, this was due to the difference in solubility, and eventual partial-solubility at the concentrations that were being used

The plotted responses for area share an almost perfect match to the amounts of organic solvents that travel towards the electrometer seen in **Section 4.2.2 Figures 26.** This is a crucial finding in the research thus far. It is apparent that the composition of the mobile phase has an effect on the response that is seen for analytes by charged aerosol detection, and this effect is directly related to that composition of the mobile phase.

Few reasons were evident as to why the albuterol had a higher response compared the other two active pharmaceutical ingredients. All conditions used for albuterol assays were exactly the same as for loratadine and mometasone furoate. Upon further examination there was one difference-the peak width. Albuterol had a broader peak when compared to the other two API's. The experimental conditions were purposely chosen to have similar peak widths upon nebulization. By not having a column any interactions with stationary phase was eliminated. These interactions would have resulted in different peak widths due to the different affinities of the analytes to the stationary phase with the varying mobile phase compositions. The only difference in the albuterol material as compared to the other two API's is that the albuterol is a salt, while the other two API's are free base materials. Potentially, upon injection the salt diffused longitudinally more readily than the albuterol resulting in a broader peak upon

detection with the charged aerosol detector. So to eliminate this variation of peak width the responses of the heights were plotted and can be seen in **Figure 30**. Once again the same profile for methanol and acetonitrile is observed for the heights, however now the albuterol responses overlay with loratadine and mometasone furoate. Similar to the previous plots by area, the responses by height for each compound also deviate from each other at higher and higher water concentrations due to differing solubilities for the concentration of the various studies.

4.2.4 Determination of Analyte Being Detected by Electrometer

The effects of mobile phase composition on HPLC-charged aerosol detection response were determined were determined by the previous experiment. For this study the amount of analyte that is actually being detected (charged) versus the amount of analyte that is going to waste was examined. The analytes used for this experiment were loratadine and mometasone furoate. These analytes were examined against the same varying compositions of mobile phase used in the previous studies. **Figures 31 through 34** contain the results obtained for the recoveries from the waste bottle.

As with the previous studies examining the percentage of water versus organic that was continuing on to the electrometer, it is easier to quantitate material that has been collected from the waste bottle rather than trying to assay the exhaust stream from the detector for the individual analytes. In this case assaying the

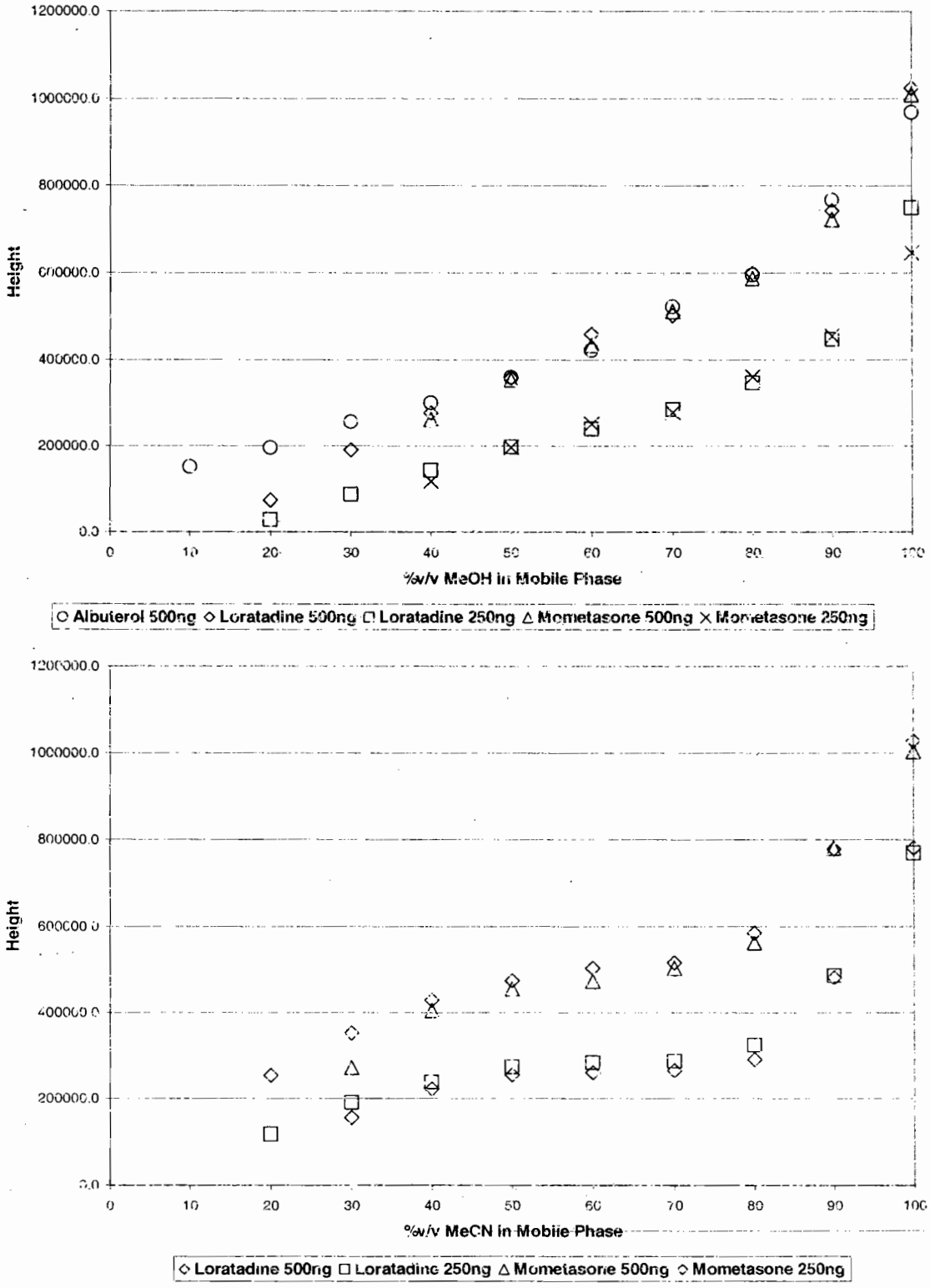


Figure 30: Height of API's versus composition of organic in mobile phase (top) methanol (bottom) acetonitrile.

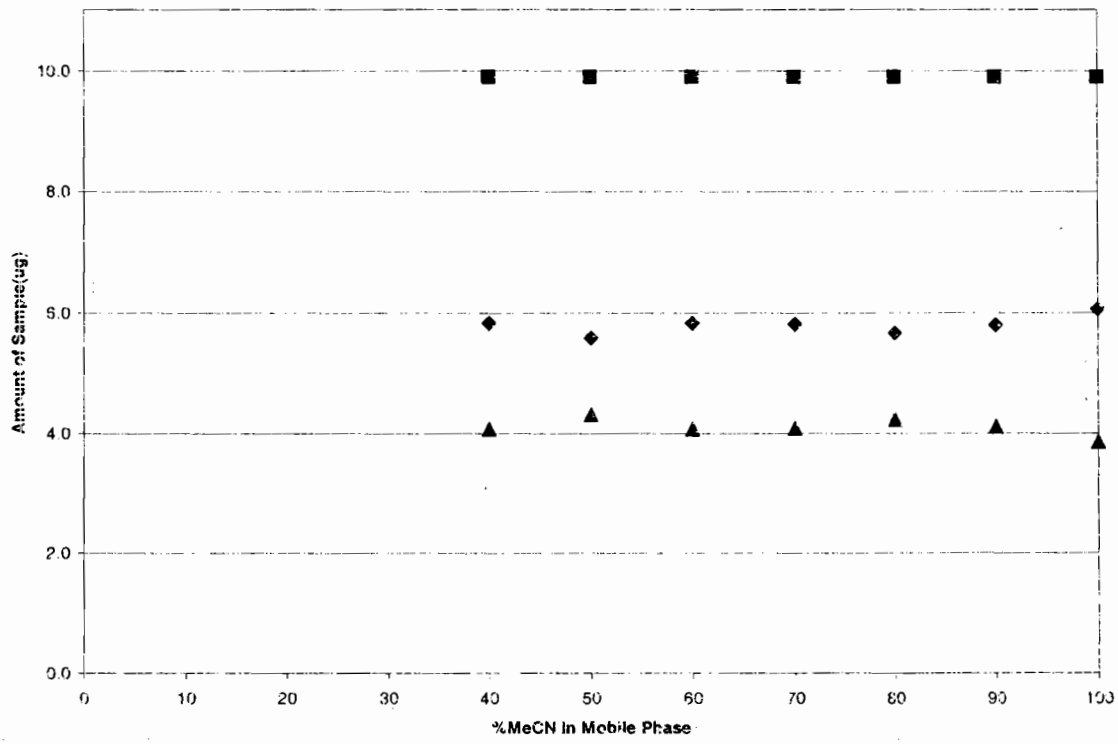


Figure 31: Loratadine recoveries for acetonitrile; amount injected (■); amount detected (♦); amount in waste (▲)

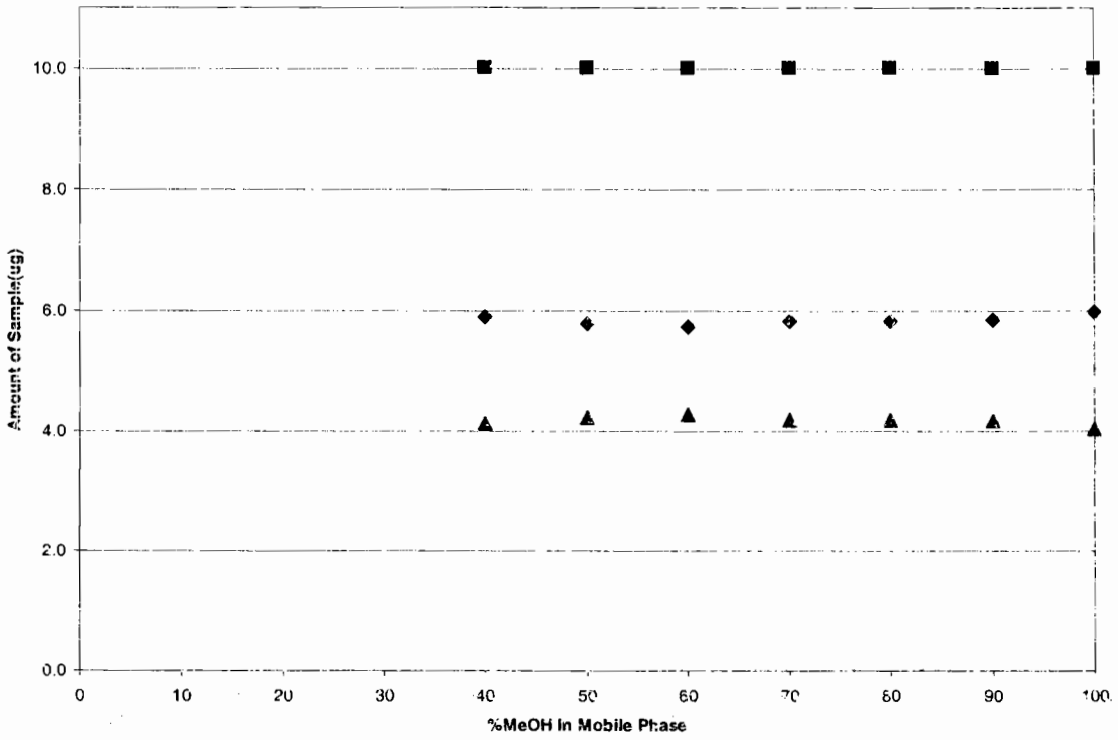


Figure 32: Loratadine recoveries for methanol; amount injected (■); amount detected (♦); amount in waste (▲)

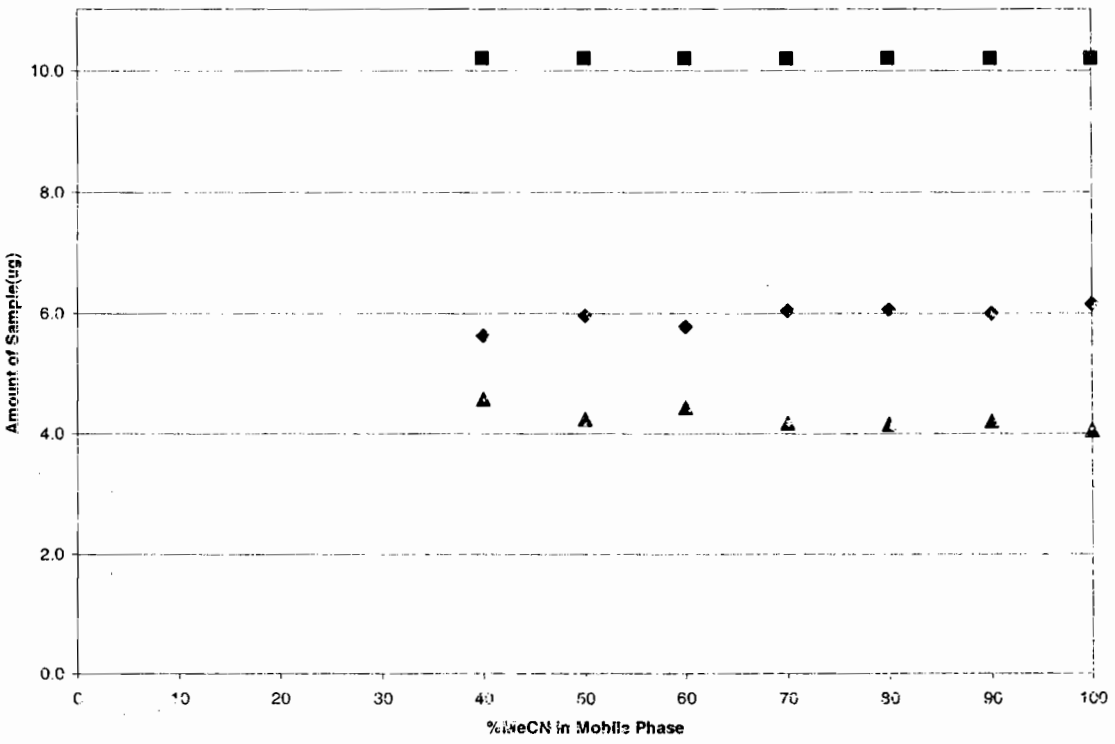


Figure 33: Mometasone Furoate recoveries for acetonitrile; amount injected (■); amount detected (♦); amount in waste (▲)

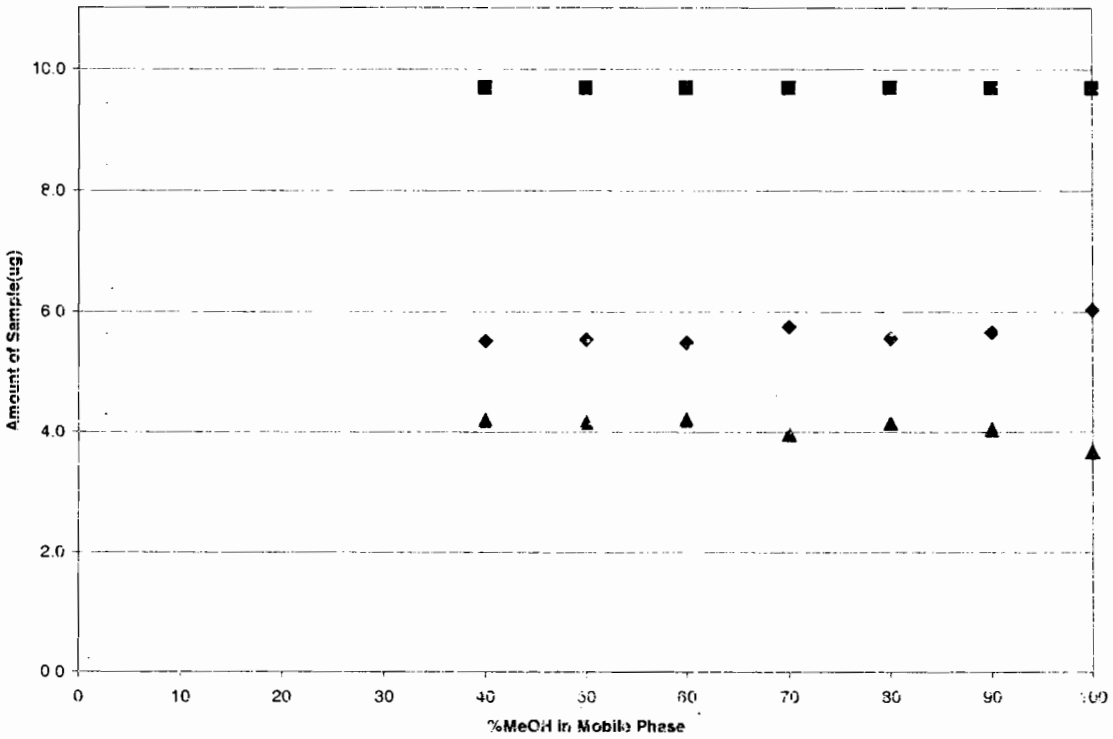


Figure 34: Mometasone Furoate recoveries for methanol; amount injected (■); amount detected (♦); amount in waste (▲)

liquid in the waste bottle for the analytes seemed to be the logical approach. Since an individual injection of analyte contains a very low level, making multiple injections increased the amount of analyte that was in the waste bottle ensuring a detectable amount of analyte. In addition to the multiple injections another concentration technique, taking a fixed volume of solution and evaporating all of the solvent away and then reconstituting it in a fixed known volume of acetonitrile was employed to further enhance the amount of analyte available for quantitation.

The results obtained for the recoveries were very similar for all four experiments. Roughly 40% of the analyte that was injected on the HPLC would go to the charged aerosol detector waste bottle, meaning that the remaining 60% of the analyte would actually be getting charged and subsequently detected by the electrometer. The experiments performed give a basic understanding of approximate analyte amounts. However the sensitivity of the analysis can be called into question. It is likely that the analytical analysis performed could not adequately detect differences in the amount of analyte that was in the waste bottle samples from one mobile phase composition change to the next. By only performing 10 injections, combined with the high degree of analyst variation from the rinse recovery technique utilized on the waste bottle leads to a high degree of analytical error for this experiment. Future work should be to re-visit this same experiment using more injections, or to determine a better technique to quantitate these values.

4.2.5 Proposed Explanation of Charged Aerosol Detection

As a point of clarification of terms to be used in this section aerosol droplets (also referred to as droplets) are what are formed from the nebulizer and consist of the analyte(s) and mobile phase. Particle(s) are the dried or partially dried droplets that move out of the drying tube. Simply droplets enter the drying tube and exit it as particles. With all of the information that has been gathered examining the charged aerosol detector, a theory can be developed explaining it's operating principles. How the charged aerosol detector actually operates, and how this might relate to the response and signal differences observed can be explained by making several assumptions. The first assumption is that upon nebulization the number of aerosol droplets are constant. For a 10 minute run time if a slice of the chromatogram were taken over any one minute interval, say from 1-2 minutes and from 5-6 minutes, they should have the same amount of aerosol droplets formed. This assumption while true for isocratic methods while may not hold true for gradient methods. As was shown in Section 4.2.2, varying the composition of the mobile phase resulted in different volumes of waste collected. This difference in volume may be attributed to a change in the number of aerosol droplets, however for a HPLC gradient method the mobile phase composition associated with the analyte upon nebulization from the start of the chromatographic peak to the end of the chromatographic peak should have no major effect on the number of droplets, assuming that a narrow Gaussian shaped peak is observed.

The next assumption is that analyte injected onto the system does not affect the number of aerosol droplets upon nebulization. While not clearly indicated in these studies, low concentration of samples (typically 0.1mg/mL) combined with small injection volumes (typically 10uL) result in dilute amounts of material in the eluate, this should have a minimal effect on the number of aerosol droplets upon nebulization. If these two assumptions are true then applying them to the actual chromatography observed will give insight to the particle formation process inside the charged aerosol detector.

Figure 35 is an example of a typical chromatographic peak. This peak has been broken up into different slices and numbered from I to IV based on the peaks similarity in concentration. In the first slice the concentration is less than in the fourth slice. This is a basic chromatographic concept that is well understood. But what happens when these bands pass through the nebulizer of the charged aerosol detector? Upon nebulization each slice passes through the nebulizer and aerosol droplets are formed at a constant rate independent of analyte concentration, see **Figure 36.** Notice that in each slice of the peak there are an identical number of aerosol droplets. While they are identical in number they are not identical in composition. The droplets have different concentrations in the different slices.

Once the aerosol droplets have been formed they move into the drying tube. Based on the mobile phase recoveries (**Section 4.2.2**), it is known that between

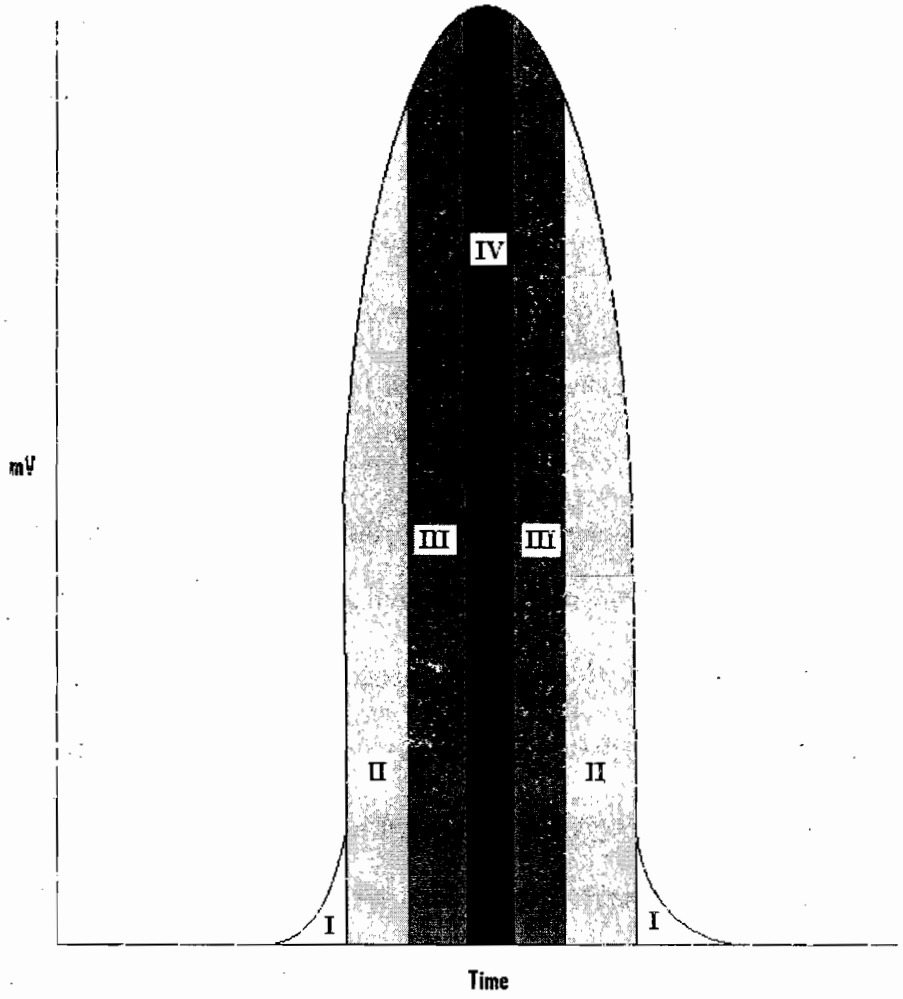


Figure 35 : An example of a typical chromatographic peak split up into concentration slices

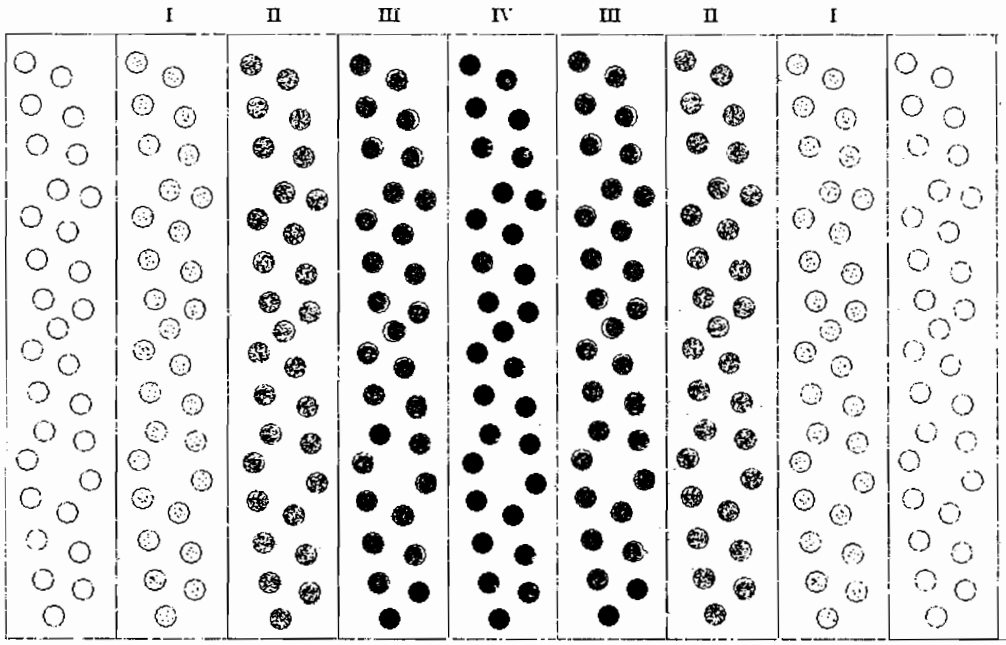


Figure 36 : An example of a typical chromatographic peak split up into concentration slices after nebulization

6 and 38% of the mobile phase actually moves into the drying tube as aerosol droplets, dependent upon the composition of the mobile phase. The drying tube is approximately 3 inches in length and is at ambient temperature, which will have a varying affect on de-solvation of the particles dependent upon mobile phase composition. Knowing if the particle is completely de-solvated or partially solvated is actually not as important as what is the size of the particle.

Additional experiments can be performed to determine the degree of particle solvation, but for the purposes of this study it is actually irrelevant. What is more important is how the compositional differences in aerosol droplets would affect the size of the particles upon charging. This can be explained going back to the nebulization assumptions, not only are there a constant number of aerosol droplets but they are also approximately the same size. For the simplification of these explanations the particles and droplets will have perfectly spherical shapes. When the aerosol droplets travel towards the charge transfer chamber they are being de-solvated at a constant rate, the droplets from the highest concentration have less solvent to lose, therefore are more likely to be solvent free particles and will have a larger diameter. The droplets from lower concentration slice will have more solvent to lose and therefore will have a smaller diameter. An example of this can be seen in **Figure 37** that shows the previous model of aerosol droplets with different concentrations and the potential difference in particles after de-solvation. The de-solvated particles in this figure were enlarged to illustrate size difference. The particle size is the most important

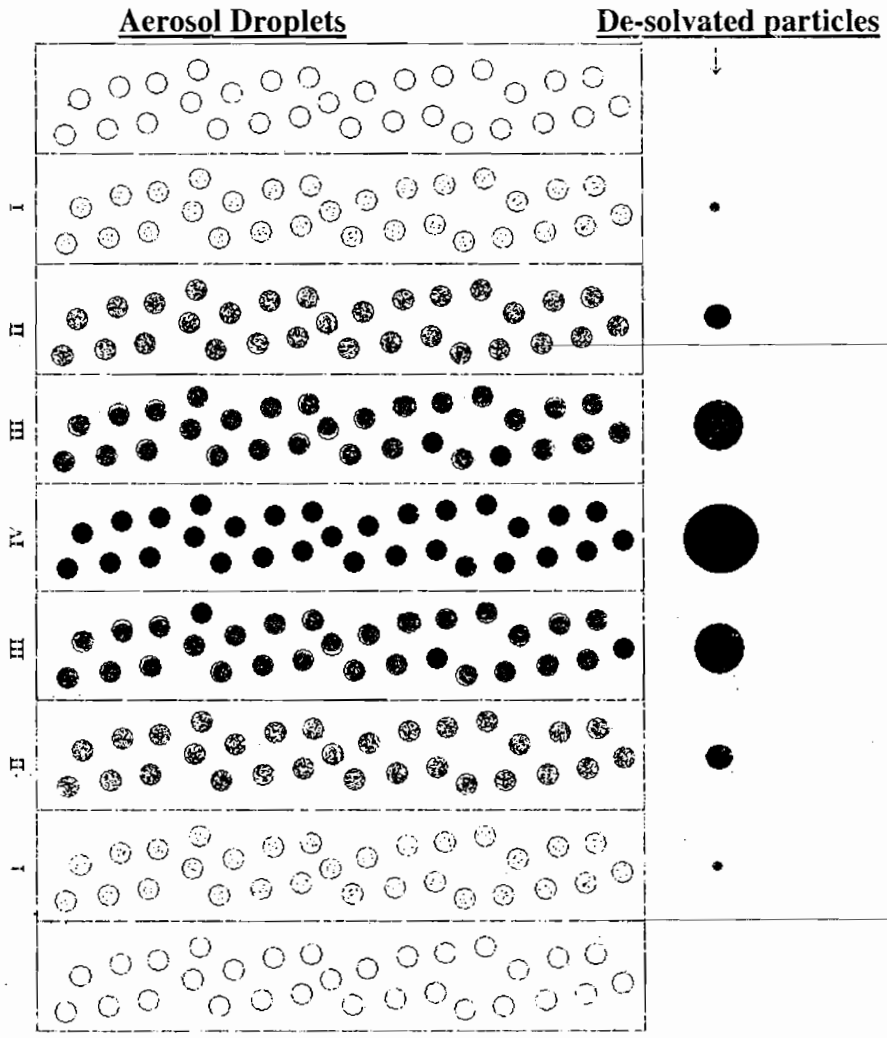


Figure 37 : An example of a typical chromatographic peak split up into concentration slices after nebulization and the subsequent de-solvated particle (de-solvated particles enlarged to illustrate size difference)

factor of this form of detection. The detector portion of the charged aerosol detector is based on TSI Inc's electrical aerosol detection technique (**Section 1.1**). The electrical aerosol detector's response is dependent on the particle's diameter. The larger the diameter of the particle, the more charges the particle can hold. Previous work by F. Kaufman has shown that using a corona-jet charger multiple charges are diffusionally transferred to the particles relative to the diameter **[57]**.

Rather than thinking in terms of diameter surface area is a more correct term. Not all particles are perfect spheres or even spheres at all, so thinking in terms of surface area is more correct. So the more surface area a particle has the more charges the particle has the potential to hold. Going back to an assumption of a perfectly spherical particle, the linear function volume was plotted against the resulting surface area for that volume, (see **Figure 38**). The response difference based on concentration of analyte for the charged aerosol detector is very similar to that of our simple volume to surface area plot. Keeping with the assumption of spherical particles, two equations from geometry help explain the response the detector gives.

Surface Area of a Sphere $$S = 4\pi r^2 \quad (7)$$

Volume of a Sphere $$V = \frac{4}{3}\pi r^3 \quad (8)$$

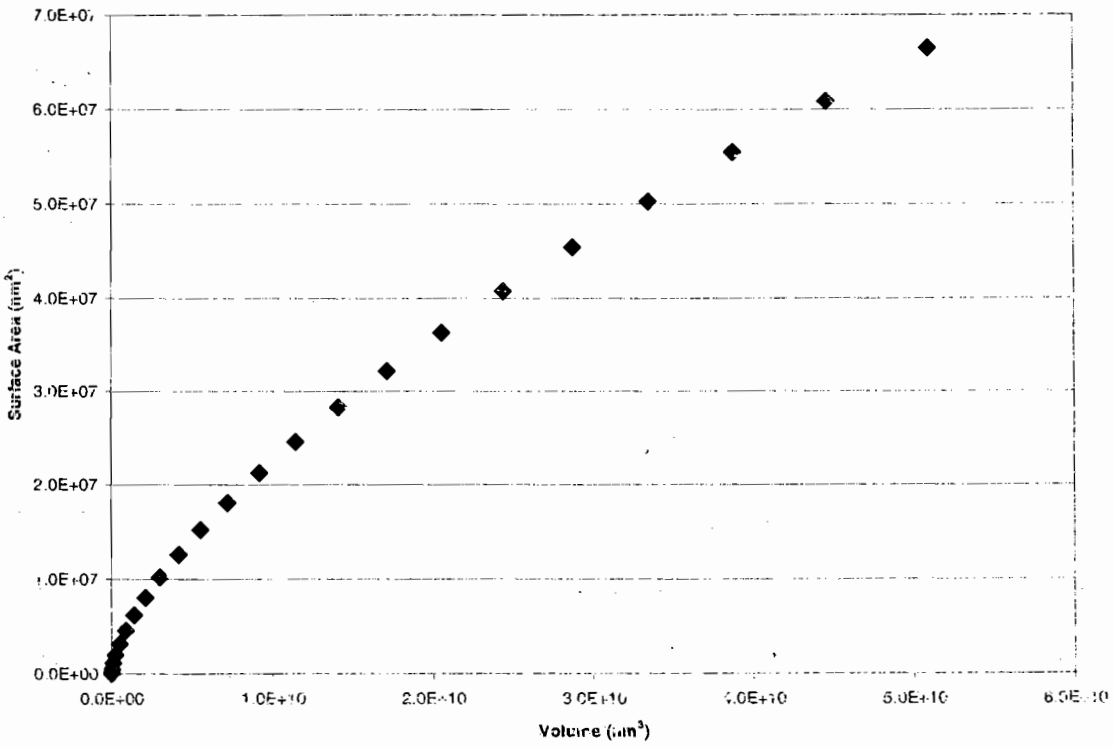


Figure 38 : Plot of theoretical particles, volume versus surface area of the resulting spherical particle

Rearranging these two equations to express volume in terms of surface area the following equation is derived:

$$V = \frac{4}{3}\pi\sqrt{\frac{S}{4\pi}}^{3} \qquad (9)$$

Equation 9 can be used to represent the response of a single particle in charged aerosol detection. The concentration, volume, is related to the surface area of that particle. The larger the surface area, the more positive charges can be diffusionally transferred onto the particle. The more charges on the particle the larger the signal that will be detected by the electrometer in the charged aerosol detector. This equation explains the response of a single particle.

In order to apply **Equation 9** to a chromatographic situation multiple particles need to be considered. At first, the chromatographic peak will be assumed to have uniform particles through the slices. The area under the peak is related to the amount of the compound that has been injected onto the system. With this model. **Equation 10** was derived.

$$Area \approx \frac{mg}{mL} \qquad (10)$$

$$Area \approx N\frac{mg}{\frac{4}{3}\pi\sqrt{\frac{S}{4\pi}}^{3}} \qquad (11)$$

Where N, a constant, is the number of particles in each slice and the peak area is being integrated from the start of the peak, a, to the end of the peak, b. This equation still does not hold true for the charged aerosol detector. The problem is with the surface area of the particles. **Equation 11** assumes that the particles are uniform, for this to be true the peak would have to be narrower than is possible for a HPLC system. In order to have a more realistic representation of the charged aerosol detector signal actual chromatographic conditions need to be considered. Band-broadening is the condition that most affects the charged aerosol detection. The initial injection onto the HPLC system is a uniform plug of liquid which travels through the system. While it moves through the system it has to deal with eddy diffusion, longitudinal diffusion, and mass transfer with the stationary phase. These effects take the initially uniform concentration of the plug of liquid injected onto the HPLC and spread that concentration out, resulting in a Gaussian shaped peak. From the examples above, upon nebulization a constant number of aerosol droplets are formed, which also means that in any given slice of the chromatographic peak a constant number of particles will also be present. The variable is the surface area of these particles, which is dependent on concentration upon nebulization, which is represented in **Equation 12**.

$$Area = \int_{a}^{b} \frac{mg}{\frac{4}{3}\pi\sqrt{\frac{S(x)}{4\pi}}^{3}} dx \qquad (12)$$

Equation 12 basically says that the Area under the peak is dependent upon the amount of charge a particle can hold based on its surface area. The amount of band broadening of the injected plug onto the HPLC system is represented by x and this variable determines the size/surface area of the particle. The assumptions that were previously made (constant number of aerosol droplets formed with a constant diameter) are ideal and can vary dependent upon mobile phase conditions as have been shown in the response experiments while varying the mobile phase composition. In an isocratic HPLC method **Equation 12** would be more accurate while in a gradient HPLC method the change in mobile phase could alter the peak area of a broad/wide peak

Sheering effects upon nebulization of the mobile phase is a possible explanation as to why the spray pattern area and aerosol droplet size can vary as a result of mobile phase changes. As illustrated in **Figure 39,** shown are two different shaded regions of the spray pattern area, one being larger than the other. Since the impactor is at a fixed position relative to the nebulizer, changes in the spray pattern area will cause different amounts of aerosol droplets to pass around the impactor and into the drying tube. This would cause an increase in signal from the detector. Sheering effects may also cause changes in aerosol droplet size upon nebulization.

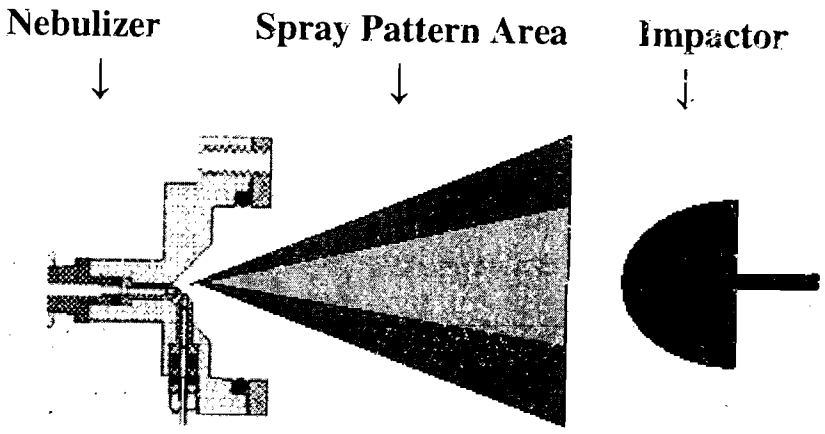


Figure 39: Diagram of spray pattern area

The surface tension of a liquid results from an imbalance of intermolecular attractive forces, a molecule at the surface of a liquid experiences only net inward cohesive forces, while molecules in the bulk liquid experience cohesive forces with other molecules in all directions **[58-60].** These forces are different based on the molecule, and when the liquid consists of varying amounts of water and methanol (or acetonitrile), the surface tension of the mobile phase will change. These cohesive forces oppose the evaporation of the liquid. Water has a higher surface tension as compared to methanol (or acetonitrile) and therefore evaporates more slowly. Surface tension could result in the increase in peak area observed. If two aerosol droplets are of uniform size and contained the same amount of analyte, one droplet that is solvated with a high content of water (say 100%) would desolvate slower then a droplet that is solvated with a high content of methanol or acetonitrile (say 100%). Individually the sheering effects upon nebulization or the surface tension of the mobile phase can explain of the results from **Section 4.2.2 and 4.2.3**. If residual solvents on the surface of the particles diminish the diffusion charge transfer, this too could explain the increase in peak area.

The experiments determining the amount of analyte being detected by the electrometer with mobile phase composition changes, **Section 4.2.4**, showed little variation as to the amount of analyte actually making its way towards the electrometer. Therefore as one trends towards higher and higher concentrations of methanol (or acetonitrile) in the mobile phase, it is less likely that the reason for the increase in peak area is related to spray pattern area changes (or aerosol

droplet size changes) from sheering effects. It is more likely that the surface tension/residual solvent associated with the particles upon diffusion charging is the reason why peak areas change when the mobile phase composition is altered. Further study and more detailed experiments to more accurately determine the amount of analyte being detected by the electrometer are required for a definitive answer.

Conclusion

Several of the mechanisms involving charged aerosol detection for high performance liquid chromatography were examined to gain an understanding of the factors that affect detection. Building on the experience gained with the initial experiments aspects of the detector were studied independent of each other. The initial findings for background comparing the data obtained for methanol and acetonitrile, indicated that the acetonitrile would be more favorable over the entire concentration range for its minimal effect on the electrometer if an assay were running a gradient. Methanol can be use in a gradient over the entire concentration range, however there will be more of a baseline rise towards the end of the gradient. Any of the mobile phases that have been examined here would be acceptable for use in isocratic mode.

The amount of mobile phase that continues past the spray chamber and does not go to the waste bottle was examined after understanding how the mobile phase alone affected detector signal. The amount of organic solvent (methanol or

acetonitrile) continuing towards the electrometer reveals a distinct but very different profile for the two organic solvents that are being examined with charged aerosol detection. The methanol shows a relatively flat response from 20% to 50%. This suggested a region of signal stability, where despite changes in the mobile phase composition the effect on an analyte will be minimized. The rest of the profile for methanol from 50% to 100% has an increasing trend, which indicated that there will be some more drastic changes of signal from an analyte due to the mobile phase composition. The acetonitrile shows a relative flat region around 50% to 90%. This suggested a region of signal stability similar to the region seen in the methanol profile. The rest of the profile for acetonitrile from 0% to 40% has an increasing trend.

Examining the response of loratadine by charged aerosol detection for several different concentrations showed this detection technique to be non-linear, which was consistent with the literature. The study of analyte charged aerosol detection response curves to changes in mobile phase showed area versus concentration plots were almost perfect matches with respect to the overall profile of the curve to the amounts of organic solvents that travel towards the electrometer. This was a crucial finding in the research thus far. It is apparent that the composition of the mobile phase has an effect on the response that is seen for analytes by charged aerosol detection, and this effect is directly related to that composition of the mobile phase. In addition while trying to understand why albuterol had a higher response compared the other two active

pharmaceutical ingredients when examining the peak area led to the discovery that peak width had an effect on the area detected by charged aerosol detection. With the peak width result for area and the previous literature and experimental results, a general equation was derived along with a simple explanation of the mechanism of charged aerosol detection works. The surface area of the particle determines the signal acquired by the electrometer for an individual particle. Furthermore, the size of the particle is dependent upon the concentration of the droplet thereby relating back to the distribution of analyte in the chromatographic band that is traveling through the HPLC system upon nebulization.

The actual amount of analyte that is making it to the electrometer for detection was determined. Roughly 40% of the analyte that was injected on the HPLC would go to the charged aerosol detector waste bottle, meaning that the remaining 60% of the analyte would actually become charged and subsequently detected by the electrometer. The experiments that were performed can give us this basic understanding of approximate analyte amounts.

Two explanations have been proposed for the detector response difference upon changes in mobile phase - sheering effects upon nebulization and surface tension of the mobile phase. The experiments determining the amount of analyte being detected by the electrometer with mobile phase composition changes showed little variation as to the amount of analyte actually making its way towards the electrometer. Therefore as one trends towards higher and higher concentrations of methanol (or acetonitrile) in the mobile phase, it is less likely

that the reason for the increase in peak area is related to spray pattern area changes (or aerosol droplet size changes) from sheering effects. It is more likely that the surface tension/residual solvent associated with the particles upon diffusion charging is the reason why peak areas change when the mobile phase composition is altered. Further study and more detailed experiments to more accurately determine the amount of analyte being detected by the electrometer are required for a definitive answer.

4.3 Properties of Mobile Phase Modifiers with HPLC-Charged Aerosol Detection

Mobile phase additives that have been examined for charged aerosol detection are very similar to those that are used in LC/MS. The additive in both forms of detection must be volatile. Three acids typically used in LC/MS (acetic, formic, and trifluoroacetic acids) were examined by HPLC-charged aerosol detection. This study was concerned with the additives effect on the baseline as well as on the signal from the analytes. With electrospray ionization (ESI) in LC/MS, trifluoroacetic acid reduces the signal in the positive ion mode by suppressing ionization and may completely suppress ionization in the negative ion mode **[61]**. This ability of trifluoroacetic acid to suppress ionization in ESI was of great interest as HPLC-charged aerosol detection is also an ionization technique.

The effect of the nature and concentration of modifier on the overall chromatography was of interest. In **Table 21** the results of the injections of the blank solutions for each additive are listed. These results were normalized based on the lowest negative data point in order to have all positive data to plot in **Figure 40**. The data for acetic acid and formic acid show that the change in baseline for the blank injections is minimal. Trifluoroacetic acid does behave differently than the other two additives. At the 0.10% concentration level trifluoroacetic acid increases the baseline dramatically.

Additive	Concentration (% by volume)	Charged Aerosol Detector Baseline Signal (mV)
None	N/A	5.00
Acetic Acid	0.01	-1.00
	0.02	-1.00
	0.05	-0.70
	0.07	-0.70
	0.10	1.30
Formic Acid	0.01	1.00
	0.02	-2.60
	0.05	-2.50
	0.07	-2.30
	0.10	9.50
Trifluoroacetic Acid	0.01	2.30
	0.02	-2.20
	0.05	-0.07
	0.07	-0.07
	0.10	65.00

Table 21: Baseline signal (mV) for mobile phase additives

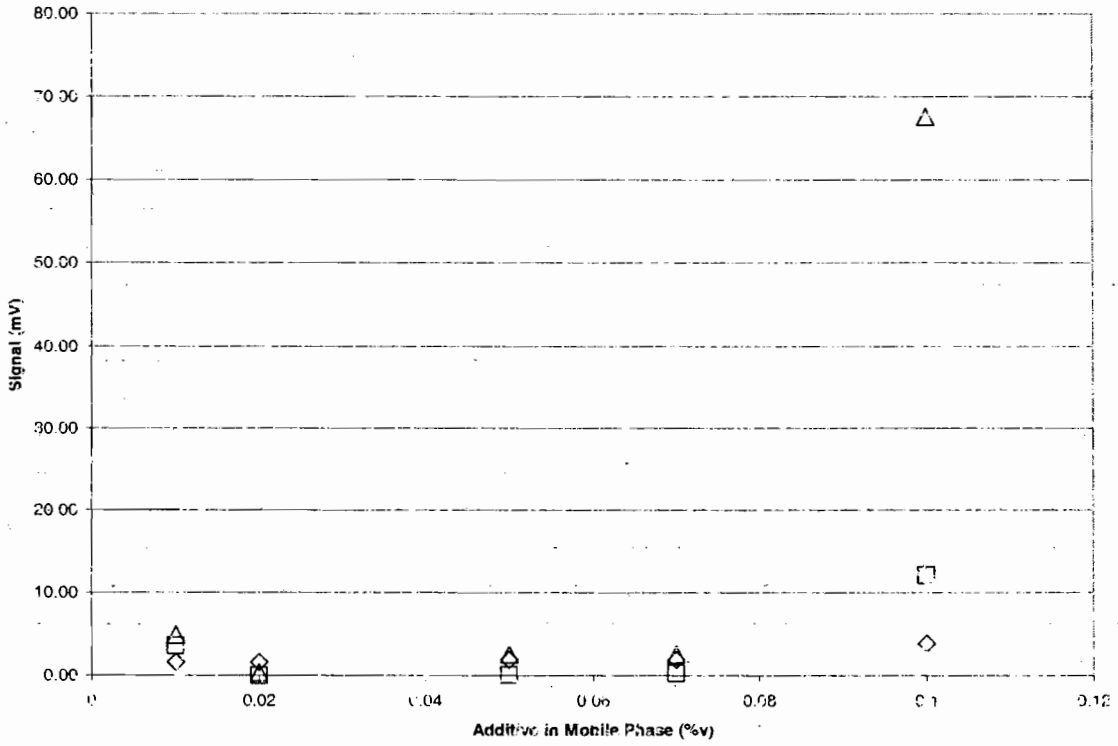


Figure 40: Baseline signal data normalized all data based on lowest result for mobile phase additives (Trifluoroacetic acid ▲, Acetic acid ♦; Formic acid ■)

In order to understand how the additives affect the signal from an analyte, the quantitation limit solution and the reference solution were injected onto the HPLC-charged aerosol detector while varying the concentration of the additives. The data that was obtained was compiled in **Table 22**. The data was compared to the initial conditions where the mobile phase had no additive. Both the quantitation limit solution and the reference solution had varying peak heights with no real pattern forming for any of the additives. There was one clear observation from the data; as a trifluoroacetic acid concentration of 0.10% by volume in the mobile phase is approached the baseline increases to a point that will affect the overall signal from the detector. **Figure 41** better illustrates this point. Also visible in this figure is the overall trend in baseline noise for the three additives. Acetic acid not only has less of a baseline rise, but also has very little noise in the baseline up to 0.10% by volume in the mobile phase. Formic acid shows somewhat of an increase in noise as the overall concentration in the mobile phase rises. Trifluoroacetic acid has the greatest increase in baseline noise out of all three of the additives. This is an important consideration when thinking about detection and quantitation limits in an analytical method. When developing a method for HPLC-charged aerosol detection it is important to check the amount of additive that is being used in the mobile phase. It is recommended that less additive can be used to achieve the same chromatographic conditions in order to maximize the detection and quantitation limits of the assay.

	Concentration (% by volume)	QL (Peak Height)	QL % Change from No Additive	Reference (Peak Height)	Reference % Change from No Additive
None	N/A	4108	N/A	509937	N/A
Acetic Acid	0.01	3723	-9.4	490937	-3.7
	0.02	3777	-8.1	469934	-7.8
	0.05	3921	-4.6	444232	-12.9
	0.07	4203	2.3	437021	-14.3
	0.10	4302	4.7	433045	-15.1
Formic Acid	0.01	4123	0.4	512784	0.6
	0.02	3520	-14.3	443403	-13.0
	0.05	3730	-9.2	414005	-18.8
	0.07	3797	-7.6	417250	-18.2
	0.10	5061	23.2	508283	-0.3
Trifluoroacetic Acid	0.01	4487	9.2	522494	2.5
	0.02	3103	-24.5	371550	-27.1
	0.05	3879	-5.6	476660	-6.5
	0.07	3406	-17.1	455844	-10.6
	0.10	2165	-47.3	383176	-24.9

Table 22: Data for QL and Reference solution injections and there % change from initial conditions with no mobile phase additive

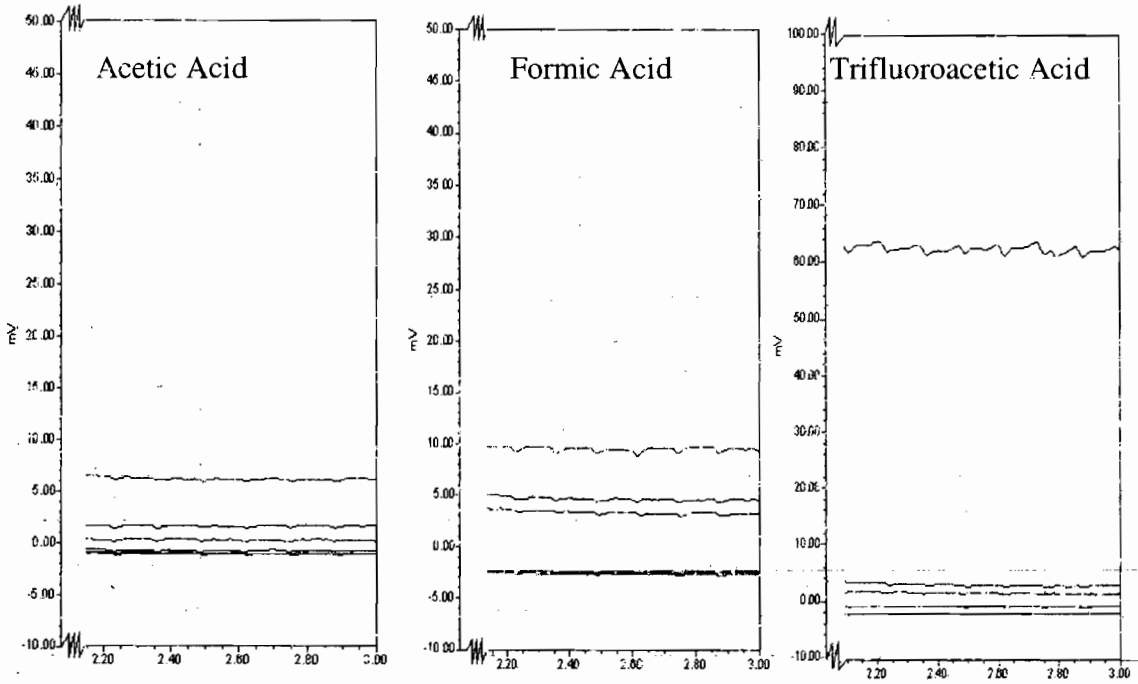


Figure 41: Effect on the chromatography of the three additives to the mobile phase

Conclusion

Volatile mobile phase additives affect the signal of an analyte in charged aerosol detector examined. The data was compared to the initial conditions where the mobile phase had no additive. There was one clear observation from the data; approaching a trifluoroacetic acid concentration of 0.10% by volume in the mobile phase increases the baseline to a point that will affect the overall signal from the detector. Acetic acid not only has less of a baseline rise, but also has very little noise in the baseline up to 0.10% by volume in the mobile phase. Formic acid shows somewhat of an increase in noise as the overall concentration in the mobile phase rises. Trifluoroacetic acid has the greatest increase in baseline noise out of all three of the additives. This is an important consideration when considering detection and quantitation limits in an analytical method. When developing a method for HPLC-charged aerosol detection it is important to check the amount of additive that is being used in the mobile phase. It is recommended that less additive be used to achieve the same chromatographic conditions in order to maximize the detection and quantitation limits of the assay.

4.4 Using HPLC-Charged Aerosol Detection for Pharmaceutical Cleaning Validation

Cleaning validation is a major analytical application in the pharmaceutical industry. HPLC with charged aerosol detection was compared and contrasted to HPLC with UV detection showing comparable performance and several advantages for charged aerosol detection, especially for analytes that do not contain a chromophore. The quantitation of drug substances is demonstrated along with the potential of charged aerosol detection for non-chromophoric excipients, providing an accurate assessment of residual contaminants remaining on the cleaned equipment. The utility of charged aerosol detection for use in pharmaceutical cleaning validation by HPLC is discussed and a practical application of this technique is described.

While HPLC with UV-Vis detection is an important means for assaying one drug substance, if impurities are present, the response at the assay wavelength for those impurities must be known and may not be the same as for the drug substance. In addition any residual material on the equipment that does not have a chromophore will not give a response. By using HPLC with charged aerosol detection, these problems are eliminated.

In this work HPLC-Charged Aerosol Detection was used to examine several traditional cleaning solvents for manufacturing devices spiked with typical drug substances and impurities (albuterol, loratadine, mometasone furoate, and lactose) (See **Figure 42**). Demonstrated was quantitation of the drug substance

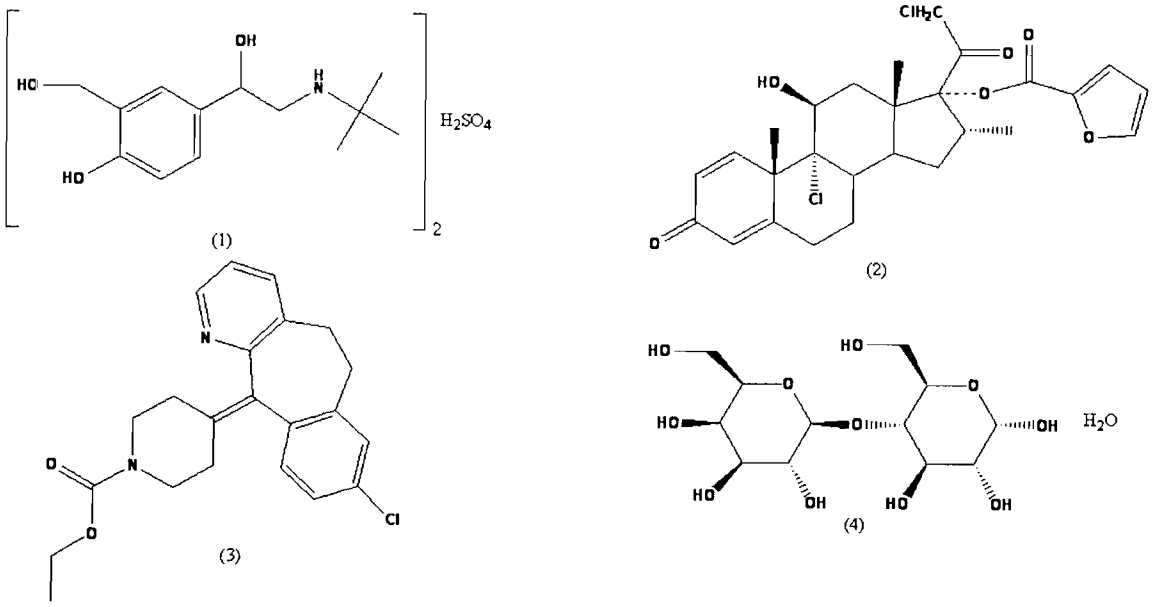


Figure 42: Compounds analyzed: albuterol (1); mometasone furoate (2); loratadine(3); lactose (4).

and examine the potential of using charged aerosol detection for non-chromophore impurities, giving an accurate picture of contaminants remaining on manufacturing equipment.

While mometasone furoate and loratadine were retained rather well on the column with a k' of 2.5 and 4.9, albuterol was not retained at all and eluted at the void volume. Quantitation of peaks in the void volume should always be avoided, but for the purpose of this study this was allowed. Solutions of varying concentrations for each drug substance were prepared and assayed on both detectors with the results presented by the calibration curves shown in **Figures 43 and 44**. The response of the UV detector varied with the molar absorptivity of the chromophores of the compounds and each compound gave a linear response. Due to different λmax's of the compounds, wavelengths of 250nm and 205nm were used. Compared to the UV detector results the charged aerosol detector showed a linear trend over the full concentration range, although it is known that charged aerosol detection gives a quadratic response over a wider concentration range **[1]**. However at this low concentration and over a small range the response can be treated as linear.

Quantitation limits were also examined for each of the three drug substances. The intention was to achieve a signal to noise ratio greater than 10 for 10ng of each drug substance on column. Both detectors were easily able to achieve this criterion, as shown in **Table 23**.

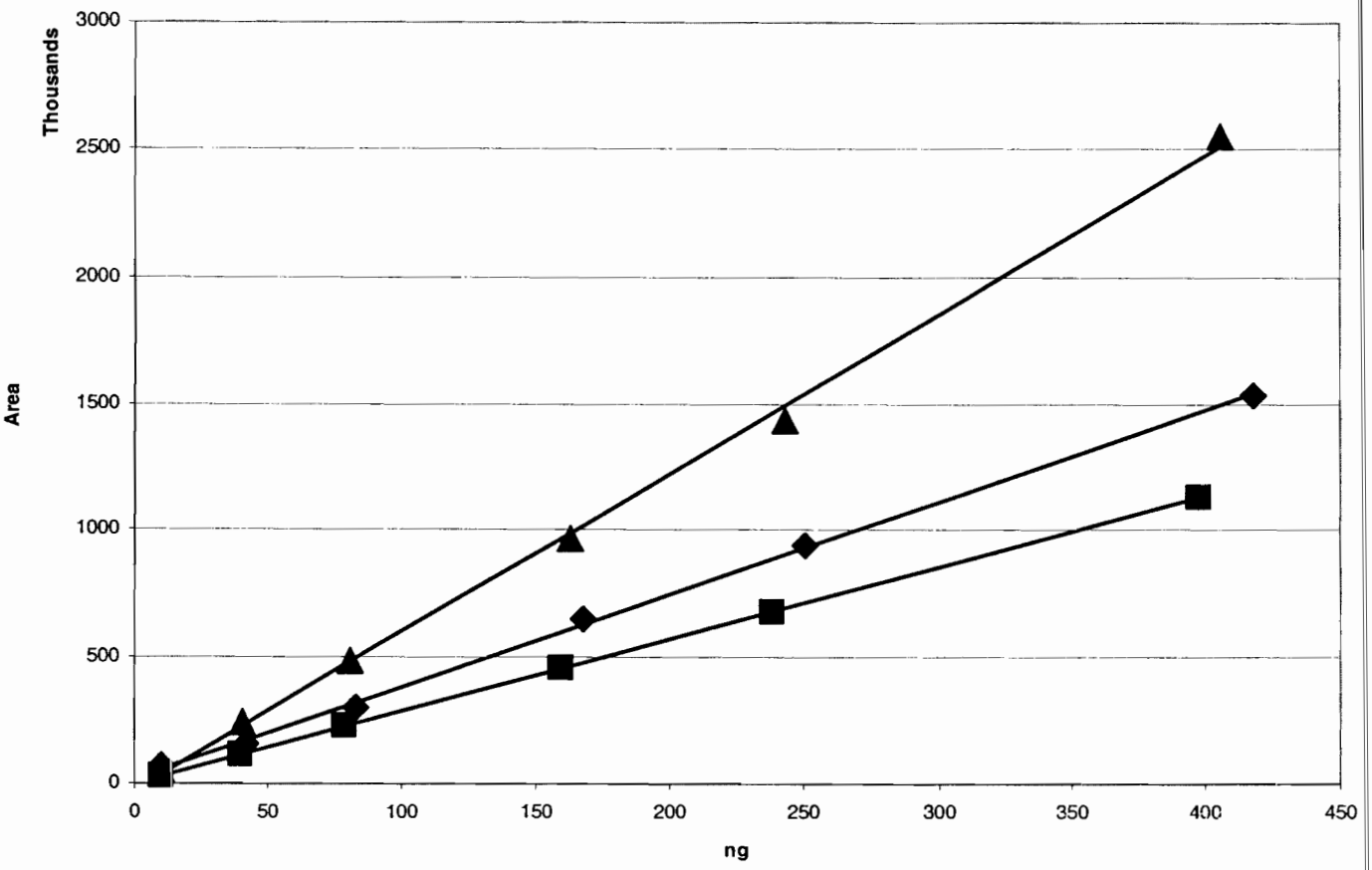


Figure 43: UV response for mometasone furoate @ 250nm (■), albuterol @ 205nm (♦), loratadine @ 205nm (▲)

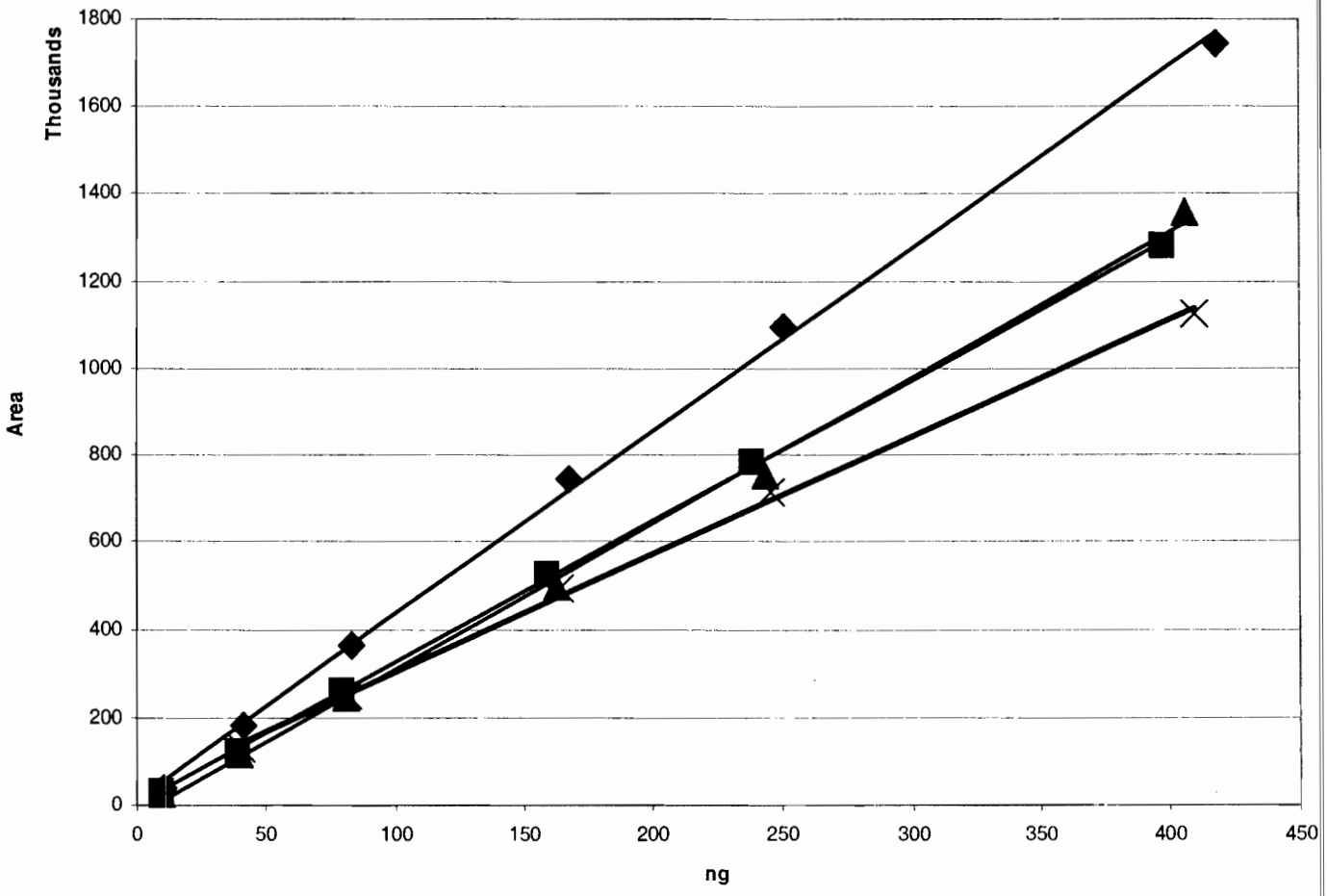


Figure 44: Charged aerosol detector response for mometasone furoate (■), albuterol (♦), loratadine(▲), lactose (x)

Compound	UV	Charged Aerosol Detector
Mometasone Furoate	17	53
Albuterol	29	20
Loratadine	35	22

Table 23: Signal to Noise for approx. 10ng injected on column

Minimization of interferences from solvents is a concern in cleaning validation. Several common cleaning solvents were selected and examined for their interferences to the chromatographic detection. The conditions mentioned above were used with one exception. The column was removed and replaced with a zero dead volume union. The solvents were injected on the system without introducing the variability of association with the stationary phase of the column. This was performed in order to get an accurate comparison of how common cleaning solvents potentially interfere with detection.

As shown in **Table 24** the UV detector at 205nm showed greater disturbance to the baseline versus charged aerosol detector for every solvent examined. Loratadine was added as a point of reference in order to show the relative differences in peak height between the UV detector at 205nm and the charged aerosol detector. The charged aerosol detector differs in response to the UV at 205nm by approximately 47% for loratadine. In each case the interference from a solvent is greater using UV at 205nm than using charged aerosol detection.

The cleaning solvents that were examined have UV cut-offs that were greater than 205nm. In addition all of these solvents have different UV responses at 205nm relative to the mobile phase. This reduction in interference from the solvents in the charged aerosol detector is due to the nebulization followed by the drying of the mobile phase. Since the solvents are volatile they are removed just as the mobile phase is removed.

	Compound	UV @ 205nm	Charged Aerosol Detector
	*Loratadine (162.4ng)	76,098	40,070
	Water	-85,319	2,825
Peak Height	Methanol	151,959	10,435
	Ethanol	905,584	48,632
	Tetrahydrofuran	1,232,235	321,012
	Acetone	1,828,765	75,548

Table 24: Solvent interference by height. *Loratadine date taken from injections from **Figures 43 & 44** and was not run under same conditions as solvents.

Several clean small stainless steel coupons measuring 2.5 x 2.5 inches with a slight bowl like indentation as shown in **Figure 45** were spiked with a known amount of three drug substances. Solvent rinse and swabbing were implemented in order to recover the drug substances from the coupons. The first and third columns in **Table 25** show the amount of material spiked onto each stainless steel coupon while the second and fourth columns show the amount that was recovered for either the rinse or the swab of the coupons. The result of the recovery study was that charged aerosol detector and UV both gave acceptable results with the charged aerosol detector giving slightly better recoveries relative to the spiked amounts versus the UV detector. The chosen excipient, lactose, does not have an effective chromophore for UV detection. In contrast, charged aerosol detection gives an excellent response. The overlay of chromatograms of lactose and a blank in **Figure 46** show a minor baseline disturbance for the blank injection and a large peak for lactose. This analysis of lactose is an excellent example of the advantage of charged aerosol detection in comparison to UV for compounds with poor to no chromophores in cleaning validation as well as other pharmaceutical applications. A comparison of the three drug substances that have previously been examined to the response for lactose assayed by charged aerosol detection can be seen in **Figure 47**. The results show a similar response for Lactose as compared to the three drug substances over the concentration range assayed.

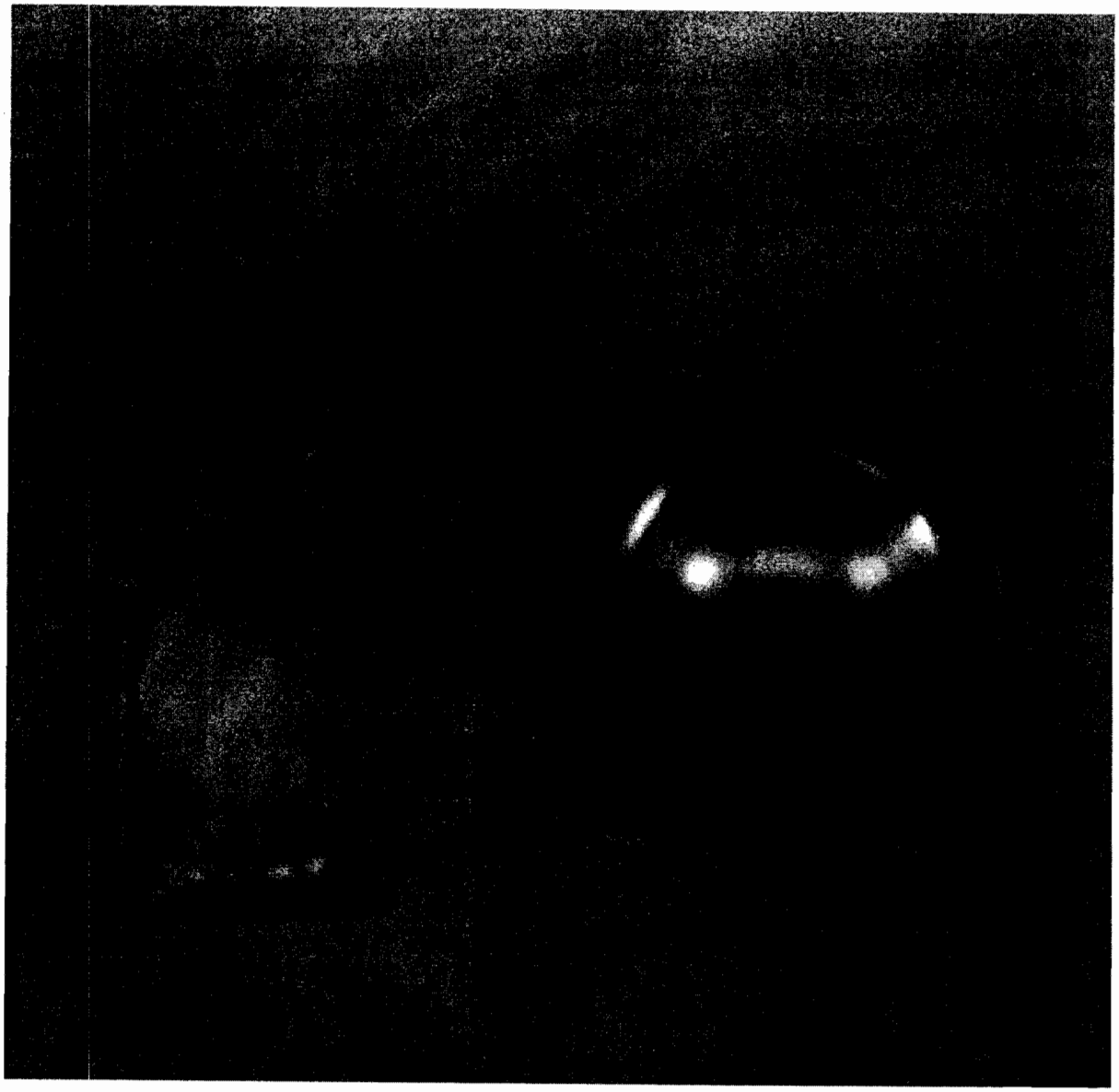


Figure 45: Photo of cotton ball swab in bottle and stainless steel coupon.

	Charged Aerosol Detector Rinse Spiked (mg)	Charged Aerosol Detector Rinse Recovered	Charged Aerosol Detector Swab Spiked (mg)	Charged Aerosol Detector Swab Recovered
Albuterol	0.960	94.5%	0.480	91.0%
Mometasone Furoate	0.966	93.2%	0.483	93.4%
Loratadine	0.930	95.2%	0.465	96.6%

	UV Rinse Spiked (mg)	UV Rinse Recovered	UV Swab Spiked (mg)	UV Swab Recovered
Albuterol	0.960	83.0%	0.480	81.0%
Mometasone Furoate	0.966	85.8%	0.483	86.7%
Loratadine	0.930	86.3%	0.465	88.6%

Table 25:. Drug substance rinsing and swabbing recovery data

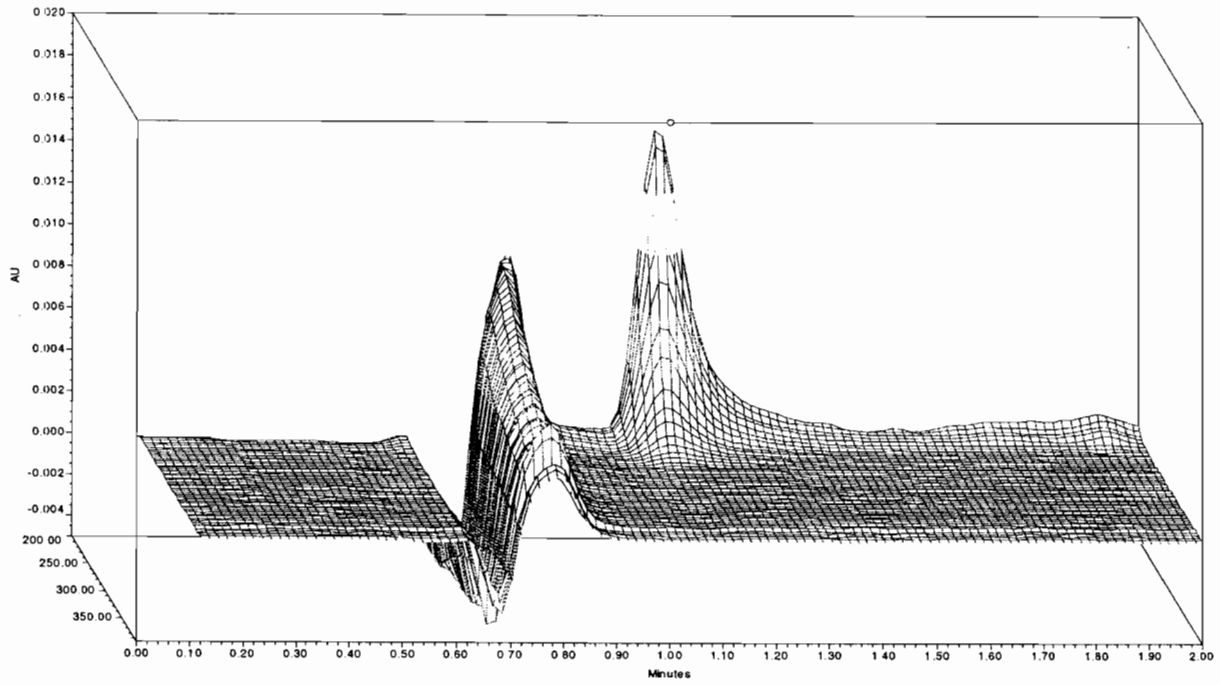


Figure 46:Photo diode array scan of lactose 410ng on column from 0 to 2 minutes

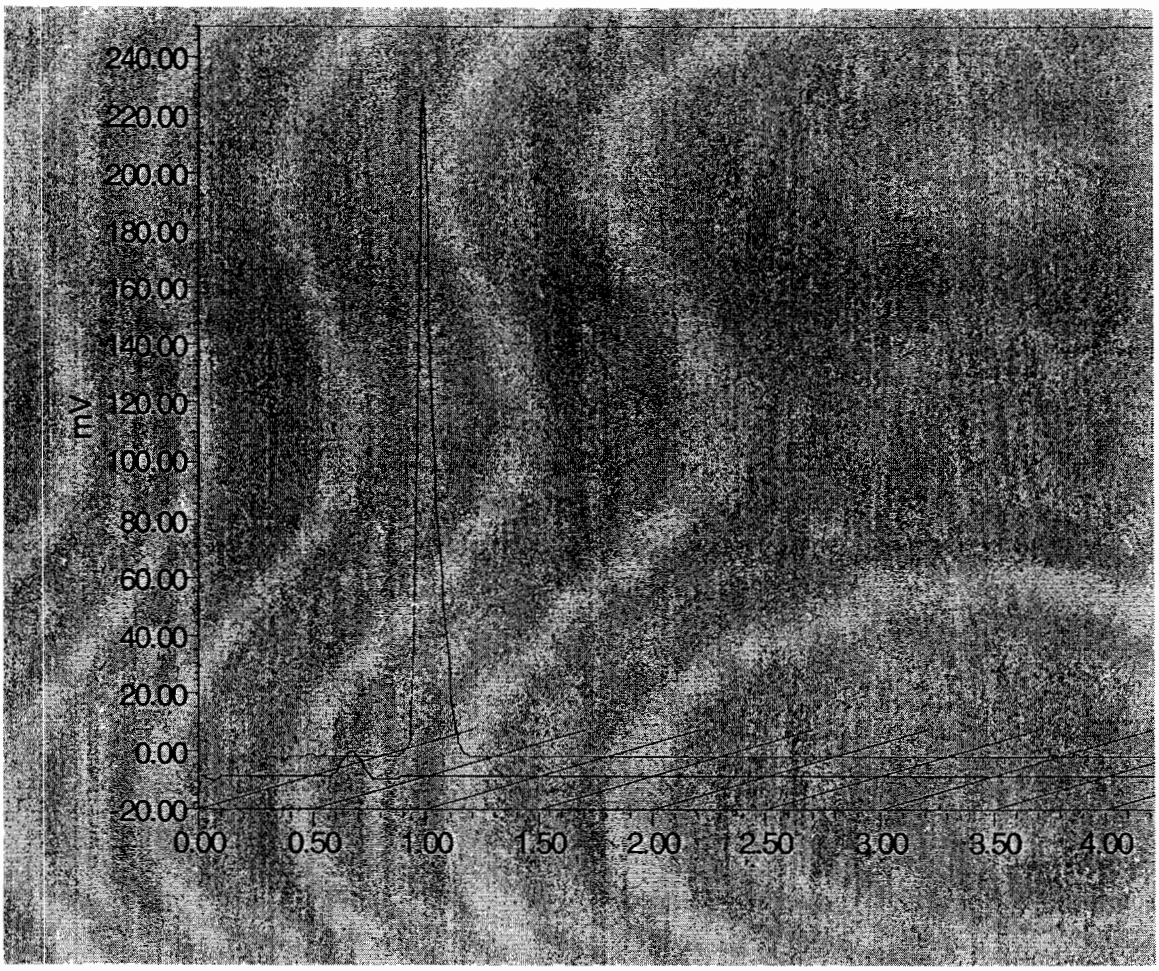


Figure 47: Charged aerosol detector overlay of lactose 410ng and blank

As with Albuterol, lactose elutes in the void volume. The response of the two compounds that did not elute in the void volume (mometasone furoate and loratadine) were consistent with each other. As for the two compounds that did elute in the void, it was observed that Albuterol has a slightly increased response compared to the retained compounds, and lactose has a slightly decreased response compared to the retained compounds. This difference in response of retained vs. un-retained is most likely due to elution in the void. Lactose was diluted in water thus when it was nebulized and dried in the detector it did not have methanol:water (75:25) surrounding it as the retained compounds it had an increased amount of water surrounding it. Similarly albuterol eluting in the void had an increased concentration of methanol surrounding it, which could lead to its slightly increased charged aerosol detector response.

Conclusion

Three drug substances and one excipient were examined by HPLC-Charged Aerosol Detection and HPLC-UV for pharmaceutical cleaning validation. Charged aerosol detection and UV-Vis detection resulted in linear curves for all three drug substances over a usable range for low level quantitation for pharmaceutical cleaning assays. Limits of quantitation were examined for the drug substances and showed equivalency between the two detection methods. Interference from the cleaning solvent was shown to be less of an issue with charged aerosol detection rather than with UV-Vis, which is advantageous when performing the assay. Recovery studies mimicking real surfaces found in

commercial equipment with two different sampling techniques used in pharmaceutical cleaning assays (rinsing, swabbing) showed the similarity between the two detectors. All of these attributes lead toward effective development and validation of HPLC-charged aerosol detector based methods for pharmaceutical cleaning validation. Examining the excipient lactose yielded the charged aerosol detector's biggest advantage over UV-Vis detection: the ability to detect and quantitate low levels of poor or non-chromophore compounds.

5. CONCLUSIONS

The validatability of a HPLC method using charged aerosol detection was examined for several different types of assays (impurity, purity, and in-process) using a hypothetical process. It was determined that these methods were fully validatable using slightly modified criteria from those typically used for HPLC method with UV detection. When deciding on which criteria to set for the charged aerosol detector, the standard validation criteria are an acceptable place to begin. However, in some cases the criteria may not be acceptable for a non-linear form of detection with a HPLC-charged aerosol detection assay. This requires good scientific judgment when drafting the validation plan. Acceptance criteria can be altered, but only to the point where the validation still proves that there are good controls in the method and that it is accurate, precise, reproducible, and robust over the range that is being utilized. By having successfully undergone pharmaceutical validation, one of the most difficult test for any analytical technique, the charged aerosol detector has been shown to be a useful and valuable detector for use with high performance liquid chromatography.

Several of the mechanisms involving charged aerosol detection for high performance liquid chromatography were examined to gain understand what may affect detection. Building on the experience gained with the initial cleaning validation experiments aspects of the detector were studied independent of each other. The initial findings for background comparing the data obtained for

methanol and acetonitrile, found that the acetonitrile would be more favorable over the entire concentration range for its minimal effect on the electrometer if an assay were running a gradient. Methanol can be use in a gradient over the entire concentration range, however there will be more of a baseline rise towards the end of the gradient. Any of the mobile phases that have been examined here would be acceptable for use in isocratic mode.

The amount of mobile phase that continues past the spray chamber and does not go to the waste bottle was examined after investigating how the mobile phase alone affected detector signal. The amount of organic solvent (methanol or acetonitrile) continuing towards the electrometer reveals a distinct but very different profile for the two organic solvents that are being examined with charged aerosol detection. Methanol shows a relatively flat response from 20% to 50%. This suggested a region of signal stability, where despite changes in the mobile phase composition the effect on an analyte will be minimized. The rest of the profile for methanol from 50% to 100% has an increasing trend, which indicated that there will be some more drastic changes of signal from an analyte due to the mobile phase composition. Acetonitrile shows a relative flat region around 50% to 90%. This suggested a region of signal stability similar to the region seen in the methanol profile. The rest of the profile for acetonitrile from 0% to 40% has an increasing trend.

Examining the response of loratadine by charged aerosol detection for several different concentrations showed this detection technique to be non-linear, which was consistent with the literature. The study of analyte charged aerosol detection response curves to changes in mobile phase showed area versus concentration plots were almost a perfect match with respect to the overall profile of the curve to the amounts of organic solvents that travel towards the electrometer. This was a crucial finding in the research thus far. It is apparent that the composition of the mobile phase has an effect on the response that is seen for analytes by charged aerosol detection, and this effect is directly related to that composition of the mobile phase. In addition while trying to understand why albuterol had a higher response compared the other two active pharmaceutical ingredients when examining the peak area led to the discovery that peak width had an effect on the area detected by charged aerosol detection. With the peak width result for area and the previous literature and experimental results, a general equation was derived along with a simple explanation of how charged aerosol detection works. The surface area of the particle determines the signal acquired by the electrometer for an individual particle. Furthermore the size of the particle is dependent upon the concentration of the droplet thereby relating back to the distribution of analyte in the chromatographic band that is traveling through the HPLC system upon nebulization.

The actual amount of analyte making it to the electrometer for detection was determined. Roughly 40% of the analyte that was injected on the HPLC would

go to the charged aerosol detector waste bottle, meaning that the remaining 60% of the analyte would actually be getting charged and subsequently detected by the electrometer. The experiments that were performed can give us this basic understanding of approximate analyte amounts. Only performing 10 injections combined with the high degree of analyst variation from the rinse recovery technique utilized on the waste bottle leads to a high degree of analytical error for this experiment. Future work should be to re-visit this same experiment using more injections, or to determine a better technique to quantitate these values.

Two explanations have been proposed for the detector response difference upon changes in mobile phase - sheering effects upon nebulization and surface tension of the mobile phase. The experiments determining the amount of analyte being detected by the electrometer with mobile phase composition changes showed little variation as to the amount of analyte actually making its way towards the electrometer. Therefore as one trends towards higher and higher concentrations of methanol (or acetonitrile) in the mobile phase, it is less likely that the reason for the increase in peak area is related to spray pattern area changes (or aerosol droplet size changes) from sheering effects. It is more likely that the surface tension/residual solvent associated with the particles upon diffusion charging is the reason why peak areas change when the mobile phase composition is altered. Further study and more detailed experiments to more accurately determine the amount of analyte being detected by the electrometer are required for a definitive answer.

Volatile mobile phase additives affect the signal of an analyte in charged aerosol detector examined. The data was compared to the initial conditions where the mobile phase had no additive. There was one clear observation from the data; approaching a trifluoroacetic acid concentration of 0.10% by volume in the mobile phase the baseline increases to a point that will affect the overall signal from the detector. Acetic acid not only has less of a baseline rise, but also has very little noise in the baseline up to 0.10% by volume in the mobile phase. Formic acid shows somewhat of an increase in noise as the overall concentration in the mobile phase rises. Trifluoroacetic acid has the greatest increase in baseline noise out of all three of the additives. This is an important consideration when considering detection and quantitation limits in an analytical method. When developing a method for HPLC-charged aerosol detection it is important to check the amount of additive that is being used in the mobile phase. It is recommended that less additive be used to achieve the same chromatographic conditions in order to maximize the detection and quantitation limits of the assay.

Three drug substances and one excipient were examined by HPLC-Charged Aerosol Detection and HPLC-UV for pharmaceutical cleaning validation. For the three drug substances assayed by charged aerosol detection and UV detection, both resulted in linear curves over a usable range for low level quantitation for pharmaceutical cleaning assays. Limits of quantitation were examined for the

drug substances and showed equivalency between the two detection methods. Interference from the cleaning solvent was shown to be less with charged aerosol detection rather than with UV which is advantageous when performing the assay. Recovery studies performed mimicking real surfaces found in commercial equipment with two different sampling techniques used in pharmaceutical cleaning assays (rinsing, swabbing) showed the similarity between the two detectors. All of these attributes lead toward effective development and validation of HPLC-charged aerosol detector based methods for pharmaceutical cleaning validation. Examining the excipient Lactose yielded the charged aerosol detector's biggest advantage over UV detection: the ability to detect and quantitate low levels of poor or non-chromophore compounds.

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